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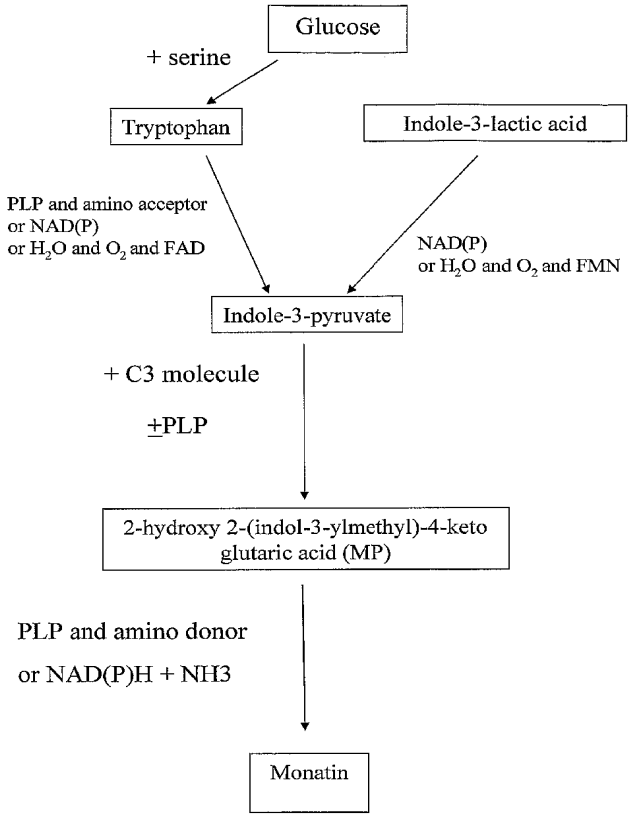
(54) Title: BEVERAGE COMPOSITIONS COMPRISING MONATIN AND METHODS OF MAKING SAME



(57) Abstract: The present invention relates to novel beverage compositions comprising monatin and methods for making such compositions. The present invention also relates to beverage compositions comprising specific monatin stereoisomers, specific blends of monatin stereoisomers, and/or monatin produced via a biosynthetic pathway *in vivo* (e.g., inside cells) or *in vitro*.

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BEVERAGE COMPOSITIONS COMPRISING MONATIN AND METHODS OF MAKING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application 60/497,627 filed August 25, 2003, the entire disclosure of which is incorporated herein by reference.

FIELD OF INVENTION

The present invention relates to novel beverage compositions comprising monatin and methods for making such compositions. The present invention also relates to beverage compositions comprising specific monatin stereoisomers, specific blends of monatin stereoisomers, and/or monatin produced via a biosynthetic pathway *in vivo* (e.g., inside cells) or *in vitro*.

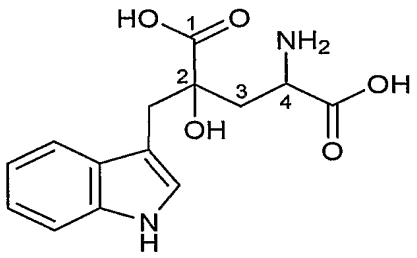
BACKGROUND

The use of non-caloric high intensity sweeteners is increasing due to health concerns raised over childhood obesity, type II diabetes, and related illnesses. Thus, a demand exists for sweeteners having a sweetness significantly higher than that in conventional sweeteners, such as granulated sugar (sucrose). Many high intensity sweeteners contain unpleasant off-flavors and/or unexpected and less-than-desirable sweetness profiles. In attempts to overcome these problems, the industry continues to conduct significant research into bitterness inhibitors, off-flavor masking technologies, and sweetener blends to achieve a sweetness profile similar to sucrose.

Monatin (2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid) is a naturally-occurring, high intensity sweetener isolated from the plant *Sclerochiton ilicifolius*, found in the Transvaal Region of South Africa. Monatin contains no carbohydrate or sugar, and nearly no calories, unlike sucrose or other nutritive sweeteners at equal sweetness.

SUMMARY

The present invention relates to beverage compositions comprising monatin (2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid—also known as 4-amino-2-hydroxy-2-(1H-indol-3-ylmethyl)-pentanedioic acid, or alternatively, based on an alternate numbering system, 4-hydroxy-4-(3-indolylmethyl) glutamic acid), a compound having the formula:



Monatin is a naturally-occurring, high intensity sweetener. Monatin has four stereoisomeric forms: 2R, 4R (the "R,R stereoisomer" or "R,R monatin"), 2S, 4S (the "S,S stereoisomer" or "S,S monatin"), 2R, 4S (the "R,S stereoisomer" or "R,S monatin"), and 2S, 4R (the "S,R stereoisomer" or "S,R monatin"). As used herein, unless stated otherwise, "monatin" refers to all four stereoisomers of monatin, as well as any blends of any combination of monatin stereoisomers (e.g., a blend of the R,R and S,S, stereoisomers of monatin).

Monatin has an excellent sweetness quality. Monatin has a flavor profile that is as clean or cleaner that other known high intensity sweeteners. The dose response curve of monatin is more linear, and therefore more similar to sucrose than other high intensity sweeteners, such as saccharin. Monatin's excellent sweetness profile makes monatin desirable for use in tabletop sweeteners, foods, beverages and other products.

Different stereoisomers of monatin, including the R,R and S,S stereoisomers, have potential in the sweetener industry, either as separate ingredients or in blends. Monatin has a desirable taste profile alone or when mixed with carbohydrates. Monatin, and blends of stereoisomers of monatin with other sweeteners, such as carbohydrates, are thought to have superior taste characteristics and/or physical qualities, as compared to other high intensity sweeteners. For example, monatin is more stable than aspartame (also known as "APM"), has a cleaner taste than saccharin, and one stereoisomer (R,R monatin) is more sweet than sucralose. Likewise, monatin sweeteners do not have the bitter aftertaste associated with saccharin, or the metallic,

acidic, astringent or throat burning aftertastes of some other high potency sweeteners. In addition, monatin sweeteners do not exhibit the licorice aftertaste associated with certain natural sweeteners, such as stevioside and glycyrrhizin.

Furthermore, unlike aspartame sweeteners, monatin sweeteners do not require a phenylalanine warning for patients with phenylketonuria. Likewise, it is expected that monatin is not cariogenic (i.e., does not promote tooth decay) because it does not contain fermentable carbohydrates. It is also expected that monatin will not cause a drop below pH ~5.7 (which can be harmful to teeth) when mixed with saliva, as measured in a pH drop test. Because of its intense sweetness, the R,R stereoisomer in particular should be economically competitive compared to other high intensity sweeteners.

In one aspect, the present invention provides a beverage composition comprising monatin or salt thereof, such as R,R, S,S, R,S or S,R monatin or a blend of different stereoisomers. As used herein, "beverage composition" refers to a composition that is drinkable as is (i.e., does not need to be diluted, or is "ready-to-drink") or a concentrate that can be diluted or mixed with a liquid to form a drinkable beverage. For example, the beverage composition can be a dry beverage mix (e.g., chocolate beverage mix, fruit beverage mix, malted beverage, or lemonade mix) that can be mixed, for example, with water or milk, to form a drinkable beverage. As another example, the beverage composition can be a beverage syrup that can be diluted, e.g., with carbonated water to form a carbonated soft drink. As another example, a beverage syrup or mix can be diluted with water/ice and one or more other ingredients (e.g., tequila) to form an alcoholic drink (e.g., a margarita). As described herein, monatin can be substituted for other common bulk sweeteners without a noticeable difference in taste. Carbonated beverages containing monatin have an improved taste profile over cola-type carbonated soft drinks sweetened with aspartame. Monatin is more stable than aspartame under acidic soft drink conditions and it is expected that monatin has a longer shelf life. As used herein, the term "carbonated" means that the beverage contains both dissolved and dispersed carbon dioxide.

In some embodiments, beverage compositions include a blend of monatin and a sweetener (e.g., sucrose or high fructose corn syrup). In other embodiments, beverage compositions comprising monatin include a flavoring, caffeine and/or a bulk sweetener. Bulk sweeteners

may be, for example, sugar sweeteners, sugarless sweeteners and lower glycemic carbohydrates (i.e., carbohydrates with a lower glycemic index than glucose). In other embodiments, monatin-containing beverage compositions include a high-intensity sweetener and/or a lower glycemic carbohydrate. In other embodiments, monatin-containing beverage compositions include a sweetness enhancer.

In some embodiments, the beverage compositions comprise monatin that consists essentially of S,S or R,R monatin. In other embodiments, the compositions contain predominantly S,S or R,R monatin. "Predominantly" means that of the monatin stereoisomers present in the composition, the monatin contains greater than 90% of a particular stereoisomer. In some embodiments, the compositions are substantially free of S,S or R,R monatin. "Substantially free" means that of the monatin stereoisomers present in the composition, the composition contains less than 2% of a particular stereoisomer. Additionally or alternatively, when used to describe monatin produced in a biosynthetic pathway, "substantially free" encompasses the amount of a stereoisomer (e.g., S,S monatin) produced as a by-product in a biosynthetic pathway involving chiral-specific enzymes (e.g., D-amino acid dehydrogenases or D-amino acid aminotransferases) and/or chiral-specific substrates (e.g., one having a carbon in the R-stereoconfiguration) to produce a different specific stereoisomer (e.g., R,R monatin)

In another aspect of the present invention, a beverage composition includes a stereoisomerically-enriched monatin mixture produced in a biosynthetic pathway. "Stereoisomerically-enriched monatin mixture" means that the mixture contains more than one monatin stereoisomer and at least 60% of the monatin stereoisomers in the mixture is a particular stereoisomer, such as R,R, S,S, S,R or R,S. In other embodiments, the mixture contains greater than 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% of a particular monatin stereoisomer. In another embodiment, a beverage composition comprises an stereoisomerically-enriched R,R or S,S monatin. "Stereoisomerically-enriched" R,R monatin means that the monatin comprises at least 60% R,R monatin. "Stereoisomerically-enriched" S,S monatin means that the monatin comprises at least 60% S,S monatin. In other embodiments, "stereoisomerically-enriched" monatin comprises greater than 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% of R,R or S,S monatin.

Monatin can be isolated from the bark of the roots of the plant *Sclerochiton ilicifolius*. For example, the bark can be ground and extracted with water, filtered and freeze dried to obtain a dark brown, amorphous mass. The mass can be re-dissolved in water and reacted with a cation resin in the acid form, e.g., "Biorad" AG50W x8 in the HCl form (Bio-Rad Laboratories, Richmond, CA). The resin can be washed with water and the compounds bound to the resin eluted using an aqueous ammonia solution. The eluate can be freeze dried and subjected to aqueous gel filtration. See, for example, U.S. Patent No. 5,128,164. Alternatively, monatin can be chemically synthesized. See, for example, the methods of Holzapfel and Olivier, Synth. Commun. 23:2511 (1993); Holzapfel et al., Synth. Commun. 38:7025 (1994); U.S. Patent No. 5,128,164; U.S. Patent No. 4,975,298; and U.S. Patent No. 5,994,559. Monatin also can be recombinantly produced.

In one aspect of the present invention, a method of making a beverage composition comprising monatin is provided. The method includes biosynthetically producing monatin either *in vivo* or *in vitro*. A "biosynthetic pathway" comprises at least one biological conversion step. In some embodiments, the biosynthetic pathway is a multi-step process and at least one step is a biological conversion step. In other embodiments, the biosynthetic pathway is a multi-step process involving both biological and chemical conversion steps. In some embodiments, the monatin produced is a stereoisomerically-enriched monatin mixture.

In another aspect of the present invention, a beverage composition comprising a biosynthetically-produced monatin is provided. Although monatin can also be chemically synthesized, biosynthetically-produced monatin may provide advantages in beverage applications over chemically-synthesized monatin because the chemically-synthesized monatin can include undesirable contaminants.

In another aspect of the present invention, several biosynthetic pathways exist for making monatin from substrates chosen from glucose, tryptophan, indole-3-lactic acid, as well as indole-3-pyruvate and 2-hydroxy 2-(indole-3-ylmethyl)-4-keto glutaric acid (also known as "the monatin precursor," "MP" or the alpha-keto form of monatin). Examples of biosynthetic pathways for producing or making monatin or its intermediates are disclosed in FIGS. 1-3 and 11-13, which show potential intermediate products and end products in boxes. For example, a conversion from one product to another, such as glucose to tryptophan, tryptophan

to indole-3-pyruvate, indole-3-pyruvate to MP, MP to monatin, or indole-3-lactic acid (indole-lactate) to indole-3-pyruvate, occurs in these pathways.

These conversions within the biosynthetic pathways can be facilitated via chemical and/or biological conversions. The term "convert" refers to the use of either chemical means or at least one polypeptide in a reaction to change a first intermediate into a second intermediate. Conversions can take place *in vivo* or *in vitro*. The term "chemical conversion" refers to a reaction that is not actively facilitated by a polypeptide. The term "biological conversion" refers to a reaction that is actively facilitated by one or more polypeptides. When biological conversions are used, the polypeptides and/or cells can be immobilized on supports such as by chemical attachment on polymer supports. The conversion can be accomplished using any reactor known to one of ordinary skill in the art, for example in a batch or a continuous reactor.

Examples of polypeptides, and their coding sequences, that can be used to perform biological conversions are shown in FIGS. 1-3 and 11-13. Polypeptides having one or more point mutations that allow the substrate specificity and/or activity of the polypeptides to be modified, can be used to make monatin. Isolated and recombinant cells expressing such polypeptides can be used to produce monatin. These cells can be any cell, such as a plant, animal, bacterial, yeast, algal, archaeal, or fungal cell.

For example, monatin-producing cells can include one or more (such as two or more, or three or more) of the following activities: tryptophan aminotransferase (EC 2.6.1.27), tyrosine (aromatic) aminotransferase (EC 2.6.1.5), tryptophan dehydrogenase (EC 1.4.1.19), glutamate dehydrogenase (EC 1.4.1.2, 1.4.1.3, 1.4.1.4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminase (EC 2.6.1.28), multiple substrate aminotransferase (EC 2.6.1.-), aspartate aminotransferase (EC 2.6.1.1), L-amino acid oxidase (EC 1.4.3.2), tryptophan oxidase (no EC number, Hadar et al., *J. Bacteriol* 125:1096-1104, 1976 and Furuya et al., *Biosci Biotechnol Biochem* 64:1486-93, 2000), D-tryptophan aminotransferase (Kohiba and Mito, Proceedings of the 8th International Symposium on Vitamin B_6 and Carbonyl Catalysis, Osaka, Japan 1990), D-amino acid dehydrogenase (EC 1.4.99.1), D-amino acid oxidase (EC 1.4.3.3), D-alanine aminotransferase (EC 2.6.1.21),

synthase/lyase (EC 4.1.3.-), such as 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16) or 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17), and/or synthase/lyase (4.1.2.-).

In another example, cells can include one or more (such as two or more, or three or more) of the following activities: indolelactate dehydrogenase (EC 1.1.1.110), R-4-hydroxyphenyllactate dehydrogenase (EC 1.1.1.222), 3-(4)-hydroxyphenylpyruvate reductase (EC 1.1.1.237), lactate dehydrogenase (EC 1.1.1.27, 1.1.1.28, 1.1.2.3), (3-imidazol-5-yl) lactate dehydrogenase (EC 1.1.1.111), lactate oxidase (EC 1.1.3.-), synthase/lyase (4.1.3.-) such as 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16) or 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17), synthase/lyase (4.1.2.-), tryptophan aminotransferase (EC 2.6.1.27), tyrosine (aromatic) aminotransferase (EC 2.6.1.5), tryptophan dehydrogenase (EC 1.4.1.19), glutamate dehydrogenase (EC 1.4.1.2, 1.4.1.3, 1.4.1.4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminase (EC 2.6.1.28), multiple substrate aminotransferase (EC 2.6.1.-), aspartate aminotransferase (EC 2.6.1.1), D-tryptophan aminotransferase, D-amino acid dehydrogenase (EC 1.4.99.1), and/or D-alanine aminotransferase (EC 2.6.1.21).

In addition, the cells can include one or more (such as two or more, or three or more) of the following activities: tryptophan aminotransferase (EC 2.6.1.27), tyrosine (aromatic) aminotransferase (EC 2.6.1.5), tryptophan dehydrogenase (EC 1.4.1.19), glutamate dehydrogenase (EC 1.4.1.2, 1.4.1.3, 1.4.1.4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminase (EC 2.6.1.28), multiple substrate aminotransferase (EC 2.6.1.-), aspartate aminotransferase (EC 2.6.1.1), L-amino acid oxidase (EC 1.4.3.2), tryptophan oxidase, D-tryptophan aminotransferase, D-amino acid dehydrogenase (EC 1.4.99.1), D-amino acid oxidase (EC 1.4.3.3), D-alanine aminotransferase (EC 2.6.1.21), indolelactate dehydrogenase (EC 1.1.1.110), R-4-hydroxyphenyllactate dehydrogenase (EC 1.1.1.222), 3-(4)-hydroxyphenylpyruvate reductase (EC 1.1.1.237), lactate dehydrogenase (EC 1.1.1.27, 1.1.1.28, 1.1.2.3), (3-imidazol-5-yl) lactate dehydrogenase (EC 1.1.1.111), lactate oxidase (EC 1.1.3.-), synthase/lyase (EC 4.1.3.-), such as 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16) or 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17), and/or synthase/lyase
(4.1.2.-).

As further example, the cells can include one or more of the following aldolase activities: KHG aldolase, ProA aldolase, KDPG aldolase and/or related polypeptides (KDPH), transcarboxybenzalpyruvate hydratase-aldolase, 4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase, trans-O-hydroxybenzylidenepyruvate hydratase-aldolase, 3-hydroxyaspartate aldolase, benzoin aldolase, dihydroneopterin aldolase, L-threo-3-phenylserine benzaldehyde-lyase (phenylserine aldolase), 4-hydroxy-2-oxovalerate aldolase, 1,2-dihydroxybenzylpyruvate aldolase, and/or 2-hydroxybenzalpyruvate aldolase.

Monatin can be produced by methods that include contacting tryptophan and/or indole-3-lactic acid with a first polypeptide, wherein the first polypeptide converts tryptophan and/or indole-3-lactic acid to indole-3-pyruvate (either the D or the L form of tryptophan or indole-3-lactic acid can be used as the substrate that is converted to indole-3-pyruvate; one of skill in the art will appreciate that the polypeptides chosen for this step ideally exhibit the appropriate specificity), contacting the resulting indole-3-pyruvate with a second polypeptide, wherein the second polypeptide converts the indole-3-pyruvate to 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid (MP), and contacting the MP with a third polypeptide, wherein the third polypeptide converts MP to monatin. Exemplary polypeptides that can be used for these conversions are shown in FIGS. 2 and 3.

Producing monatin in a biosynthetic pathway via one or more biological conversions provides certain advantages. For example, by using specific polypeptides and/or certain substrates in the biosynthetic pathway, one can produce a mixture enriched in a specific stereoisomer, and/or produce a monatin mixture that is substantially free of one or more stereoisomers.

A monatin composition may include impurities as a consequence of the method used for monatin synthesis. Monatin compositions produced by purely synthetic means (i.e., not involving at least one biological conversion) will contain different impurities than monatin compositions produced via a biosynthetic pathway. For example, based on raw materials used, monatin compositions produced by purely synthetic means may include petrochemical, toxic and/or other hazardous contaminants inappropriate for human consumption. Examples of such contaminants are hazardous chemicals, such as LDA, hydrogen-Pd/C, diazomethane, KCN, Grignard's reagent and Na/Hg. On the other hand, it is expected that a monatin

composition produced via a biosynthetic pathway may contain edible or potable impurities, but will not contain petrochemical, toxic and/or other hazardous material.

It is expected that a method for producing monatin in a biosynthetic pathway via one or more biological conversions produces fewer toxic or hazardous contaminants and/or can provide a higher percentage of a particular stereoisomer, as compared to purely synthetic means. For example, it is expected that when making monatin using D-amino acid dehydrogenases, D-alanine (aspartate) aminotransferases, D-aromatic aminotransferases or D-methionine aminotranferases, one can obtain at least 60% R,R monatin and less than 40 % S,S, S,R and/or R,S monatin. It is also expected, for example, that when making monatin using the above-mentioned D-enzymes, as well as at least one substrate (e.g., the monatin precursor) having a carbon in the R-stereoconfiguration, one can obtain at least 95 % R,R monatin and less than 5% S,S, S,R and/or R,S monatin. In contrast, it is expected that when making monatin by purely synthetic means, one obtains about 25%-50% of the desired stereoisomer.

In one embodiment, a method for producing monatin via a biosynthetic pathway, for example, involving one or more biological conversion, produces no petrochemical, toxic or hazardous contaminants. "Petrochemical, toxic or hazardous contaminants" means any material that is petrochemical, toxic, hazardous and/or otherwise inappropriate for human consumption, including those contaminants provided as raw material or created when producing monatin via purely synthetic means. In another embodiment, a method for producing monatin via a biosynthetic pathway, for example, involving one or more biological conversion, produces only edible or potable material. "Edible or potable material" means one or more compounds or material that are fit for eating or drinking by humans, or otherwise safe for human consumption. Examples of edible or potable material include monatin, tryptophan, pyruvate, glutamate, other amino acids, as well as other compounds or material that are naturally present in the body.

In one embodiment, a beverage composition comprising monatin or salt thereof contains less calories and carbohydrates than the same amount of the beverage composition containing sucrose or high fructose corn syrup in place of the monatin or salt thereof at comparable sweetness. "A sweetness comparable" or "comparable sweetness" means that an experienced sensory evaluator, on average, will determine that the sweetness presented in a first

composition is within a range of 80% to 120% of the sweetness presented a second composition.

In other embodiments, a beverage composition comprising monatin or salt thereof further comprises a citrus flavor, wherein the monatin or salt thereof is present in an amount that enhances the flavor provided by the citrus flavor. In another embodiment, the beverage composition further comprises a citrus flavor and a carbohydrate, and wherein the monatin or salt thereof and the carbohydrate are present in an amount that enhances the flavor provided by the citrus flavor. The carbohydrate may be chosen from, but is not limited to, erythritol, maltodextrin, sucrose and a combination thereof.

In one embodiment, a carbonated beverage comprises a syrup composition in an amount ranging from about 15% to about 25% by weight of the carbonated beverage, wherein the syrup composition comprises monatin or salt thereof.

In another embodiment, a beverage composition comprises from about 3 to about 10000 ppm monatin or salt thereof. In other embodiments, the beverage composition comprises from about 3 to less than about 30 ppm monatin, or from more than 2500 to about 10000 ppm monatin. In another embodiment, a beverage composition is a syrup or dry beverage mix, wherein the composition comprises from about 10 to about 10000 ppm monatin or salt thereof. For example, the beverage composition can be a syrup that is a concentrate adapted for dilution in a drink in a range of about 1 part syrup:3 parts drink to about 1 part syrup:5.5 drink. In one embodiment, the syrup comprises from about 600 to about 10000 ppm S,S monatin or salt thereof. In another embodiment, the syrup comprises from about 18 to about 300 ppm R,R monatin or salt thereof. Alternatively, the syrup comprises from about 0 to about 10000 ppm S,S monatin or salt thereof, and from 0 to about 300 ppm R,R monatin or salt thereof.

In another embodiment, a beverage composition is a dry beverage mix comprising from about 10 to about 10000 ppm monatin or salt thereof. In one embodiment, the dry beverage mix comprises from about 600 to about 10000 ppm S,S monatin or salt thereof. In another embodiment, the dry beverage mix comprises from about 10 to about 450 ppm R,R monatin or salt thereof. Alternatively, the dry beverage mix comprises from about 0 to about 10000

ppm S,S monatin or salt thereof, and from about 0 to about 450 ppm R,R monatin or salt thereof.

In other embodiments, a beverage composition comprises from about 3 to about 10000 ppm monatin or salt thereof, and the composition is substantially free of R,R monatin or salt thereof, or is substantially free of S,S monatin or salt thereof. In another embodiment, a beverage composition comprises from about 3 to about 450 ppm R,R monatin or salt thereof (e.g., from about 6 to about 225 ppm R,R monatin or salt thereof). In another embodiment, a beverage composition comprises from about 3 to about 10000 ppm S,S monatin or salt thereof (e.g., from about 60 to about 4600 ppm of S,S monatin or salt thereof). In another embodiment, a beverage composition comprises from about 0 to about 10000 ppm of S,S monatin or salt thereof, and from about 0 to about 450 ppm R,R monatin or salt thereof.

In one embodiment, a beverage composition is a ready-to-drink composition comprising from about 3 to about 2000 ppm monatin or salt thereof. In another embodiments, the ready-to-drink composition comprises from about 5 to about 50 ppm R,R monatin or salt thereof, or from about 60 to about 2000 ppm S,S monatin or salt thereof.

In another embodiment, a beverage composition comprises about 450 or less ppm R,R monatin or salt thereof, and is substantially free of S,S, S,R or R,S monatin or salt thereof. Alternatively, a beverage composition comprises about 10000 or less ppm S,S monatin or salt thereof, and is substantially free of R,R, S,R or R,S monatin or salt thereof. In some embodiments, the monatin or salt thereof in a beverage composition consists essentially of R,R monatin or salt thereof, or consists essentially of S,S monatin or salt thereof. In other embodiments, the monatin or salt thereof in a beverage composition is a stereoisomerically-enriched R,R monatin or salt thereof, or is a stereoisomerically-enriched S,S monatin or salt thereof. In other embodiments, the monatin or salt thereof in a beverage composition comprises at least 95% R,R monatin or salt thereof, or at least 95% S,S monatin or salt thereof.

In one embodiment, a beverage composition comprises monatin or salt thereof that is produced in a biosynthetic pathway. In another embodiment, a beverage composition comprises a stereoisomerically-enriched monatin mixture, wherein the monatin mixture is

produced via a biosynthetic pathway. In one embodiment, the biosynthetic pathway is a multi-step pathway and at least one step of the multi-step pathway is a chemical conversion. In other embodiments, the monatin mixture produced via a biosynthetic pathway is predominantly R,R monatin or salt thereof, or is predominantly S,S monatin or salt thereof.

In one embodiment, a beverage composition comprises a monatin composition produced in a biosynthetic pathway, wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants. In another embodiment, a beverage composition comprises monatin or salt thereof, wherein the monatin or salt thereof is produced in a biosynthetic pathway and isolated from a recombinant cell, and wherein the recombinant cell does not contain petrochemical, toxic or hazardous contaminants.

In one embodiment, a beverage composition comprising monatin or salt thereof is non-cariogenic. In other embodiments, a beverage composition comprising monatin or salt thereof further comprises erythritol, trehalose, a cyclamate, D-tagatose or combination thereof.

In other embodiments, a beverage composition comprising monatin or salt thereof further comprises a bulk sweetener, a high-intensity sweetener, a lower glycemic carbohydrate, a flavoring, an antioxidant, caffeine, a sweetness enhancer or a combination thereof. For example, the flavoring may be chosen from a cola flavor, a citrus flavor and a combination thereof. For example, the bulk sweetener may be chosen from corn sweeteners, sucrose, dextrose, invert sugar, maltose, dextrin, maltodextrin, fructose, levulose, high fructose corn syrup, corn syrup solids, levulose, galactose, trehalose, isomaltulose, fructo-oligosaccharides and a combination thereof. For example, the high-intensity sweetener may be chosen from sucralose, aspartame, saccharin, acesulfame K, alitame, thaumatin, dihydrochalcones, neotame, cyclamates, stevioside, mogroside, glycyrrhizin, phyllodulcin, monellin, mabinlin, brazzein, circulin, pentadin and a combination thereof. For example, the lower glycemic carbohydrate may be chosen from D-tagatose, sorbitol, mannitol, xylitol, lactitol, erythritol, maltitol, hydrogenated starch hydrolysates, isomalt, D-psicose, 1,5 anhydro D-fructose and a combination thereof. For example, the sweetness enhancer may be chosen from curculin, miraculin, cynarin, chlorogenic acid, caffeic acid, strogins, arabinogalactan, maltol, dihyroxybenzoic acids and a combination thereof.

In another embodiment, a beverage composition comprises monatin or salt thereof that is a blend of R,R and S,S, monatin or salt thereof. In addition, a beverage composition may comprises a blend of monatin or salt thereof and a non-monatin sweetener. Non-monatin sweetener may be chosen from, for example, sucrose and high fructose corn syrup.

In some embodiments, methods for making a beverage composition comprising monatin or salt thereof comprise producing monatin or salt thereof from at least one substrate chosen from glucose, tryptophan, indole-3-lactic acid, indole-3-pyruvate and the monatin precursor. The methods may further comprise combining the monatin or salt thereof with at least one other ingredient that is not monatin or salt thereof (e.g., erythritol, trehalose, a cyclamate, D-tagatose, maltodextrin or combination thereof). In some embodiments, the other ingredient may be chosen from, for example, bulking agents, bulk sweeteners, liquid sweeteners, lower glycemic carbohydrates, high intensity sweeteners, thickeners, fats, oils, emulsifiers, antioxidants, sweetness enhancers, colorants, flavorings, caffeine, acids, powders, flow agents, buffers, protein sources, flavor enhancers, flavor stabilizers and a combination thereof. The bulk sweeteners may be chosen from, for example, sugar sweeteners, sugarless sweeteners, lower glycemic carbohydrates and a combination thereof. In other embodiments, beverage compositions made by the methods comprise from about 0 to about 10000 ppm of S,S monatin or salt thereof, and from about 0 to about 450 ppm R,R monatin or salt thereof.

In other embodiments, methods for making a beverage composition comprising monatin or salt thereof comprise producing monatin or salt thereof through a biosynthetic pathway. In some embodiments, methods for making a beverage composition comprising monatin or salt thereof comprise producing monatin or salt thereof using at least one biological conversion, or using only biological conversions. In another embodiment, a method for making a beverage composition comprising a monatin composition comprises: (a) producing monatin or salt thereof in a biosynthetic pathway in a recombinant cell; (b) isolating the monatin composition from the recombinant cell, wherein the monatin composition consists of monatin or salt thereof and other edible or potable material.

In other embodiments, a method for making a beverage composition comprising a monatin composition comprises producing the monatin composition in a biosynthetic pathway,

wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants. In other embodiments, a method for making a beverage composition comprising a monatin composition comprises producing the monatin composition from a substrate in a multi-step pathway, wherein one or more steps in the multi-step pathway is a biological conversion, and wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants.

In other embodiment, a method for making a beverage composition comprising a monatin composition comprises producing the monatin composition in a biosynthetic pathway, wherein the monatin composition consists of monatin or salt thereof and other edible or potable material. In another embodiment, a method for making a beverage composition comprising a monatin composition comprises producing the monatin composition from a substrate in a multi-step pathway, wherein one or more steps in the multi-step pathway is a biological conversion, and wherein the monatin composition consists of monatin or salt thereof and other edible or potable material.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

It will be apparent to one of ordinary skill in the art from the teachings herein that specific embodiments of the present invention may be directed to one or a combination of the above-indicated aspects, as well as other aspects. Other features and advantages of the invention will be apparent from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows biosynthetic pathways used to produce monatin and/or indole-3-pyruvate. One pathway produces indole-3-pyruvate via tryptophan, while another produces indole-3-pyruvate via indole-3-lactic acid. Monatin is subsequently produced via a MP intermediate.

Compounds shown in boxes are substrates and products produced in the biosynthetic pathways. Compositions adjacent to the arrows are cofactors, or reactants that can be used during the conversion of a substrate to a product. The cofactor or reactant used will depend upon the polypeptide used for the particular step of the biosynthetic pathway. The cofactor PLP (pyridoxal 5' – phosphate) can catalyze reactions independent of a polypeptide, and therefore, merely providing PLP can allow for the progression from substrate to product.

FIG. 2 is a more detailed diagram of the biosynthetic pathway that utilizes the MP intermediate. The substrates for each step in the pathways are shown in boxes. The polypeptides allowing for the conversion between substrates are listed adjacent to the arrows between the substrates. Each polypeptide is described by its common name and an enzymatic class (EC) number.

FIG. 3 shows a more detailed diagram of the biosynthetic pathway of the conversion of indole-3-lactic acid to indole-3-pyruvate. The substrates are shown in boxes, and the polypeptides allowing for the conversion between the substrates are listed adjacent to the arrow between the substrates. Each polypeptide is described by its common name and an EC number.

FIG. 4 shows one possible reaction for making MP via chemical means.

FIGS. 5A and 5B are chromatograms showing the LC/MS identification of monatin produced enzymatically.

FIG. 6 is an electrospray mass spectrum of enzymatically synthesized monatin.

FIGS. 7A and 7B are chromatograms of the LC/MS/MS daughter ion analyses of monatin produced in an enzymatic mixture.

FIG. 8 is a chromatogram showing the high-resolution mass measurement of monatin produced enzymatically.

FIGS. 9A-9C are chromatograms showing the chiral separation of (A) R-tryptophan, (B) S-tryptophan, and (C) monatin produced enzymatically.

FIG. 10 is a bar graph showing the relative amount of monatin produced in bacterial cells following IPTG induction. The (-) indicates a lack of substrate addition (no tryptophan or pyruvate was added).

FIGS. 11-12 are schematic diagrams showing pathways used to increase the yield of monatin produced from tryptophan or indole-3-pyruvate.

FIG. 13 is a schematic diagram showing a pathway that can be used to increase the yield of monatin produced from tryptophan or indole-3-pyruvate.

FIG. 14 presents a dose response curve obtained with an R,R, stereoisomer of monatin.

FIG. 15 presents a dose response curve obtained with an R,R/S,S stereoisomer mixture of monatin.

FIG. 16 compares the dose response curve obtained with an R,R/S,S stereoisomer mixture of monatin to a dose response curve obtained with saccharin.

FIG. 17 shows reversed phase chromatography of standards of synthetically produced monatin.

FIG. 18 shows chiral chromatography of monatin standards.

DETAILED DESCRIPTION

Overview of Biosynthetic pathways for Monatin Production

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. As used herein, "including" means "comprising." In addition, the singular forms "a" or "an" or "the" include plural references unless the context clearly dictates otherwise. The term "about" encompasses the range of experimental error that occurs in any measurement. Unless otherwise stated, all measurement numbers are presumed to have the word "about" in front of them even if the word "about" is not expressly used. The term "% wt/vol" or "% w/v" refers to percentage weight per volume, where 100% wt/vol is 1 g/mL. Thus, for example, 1 g/100 mL is 1% wt/vol (in liquid compositions). The term "ppm" refers to parts per million. Eighty ppm of monatin, for example, means 80 grams (g) of monatin in a million grams. Likewise, 1 ppm = 0.0001 % w/w or, for aqueous solutions, = 1 mg/L = 1 μg/mL = 0.0001 % wt/vol.

As shown in FIGS. 1–3 and 11-13, many biosynthetic pathways can be used to produce monatin or its intermediates such as indole-3-pyruvate or MP. For the conversion of each substrate (e.g., glucose, tryptophan, indole-3-lactic acid, indole-3-pyruvate, and MP) to each product (e.g., tryptophan, indole-3-pyruvate, MP and monatin), several different polypeptides can be used. Moreover, these reactions can be carried out *in vivo*, *in vitro*, or through a combination of *in vivo* reactions and *in vitro* reactions, such as *in vitro* reactions that include non-enzymatic chemical reactions. Therefore, FIGS. 1-3 and 11-13 are exemplary, and show multiple different pathways that can be used to obtain desired products.

Glucose to Tryptophan

Many organisms can synthesize tryptophan from glucose. The construct(s) containing the gene(s) necessary to produce monatin, MP, and/or indole-3-pyruvate from glucose and/or tryptophan can be cloned into such organisms. It is shown herein that tryptophan can be converted into monatin.

In other examples, an organism can be engineered using known polypeptides to produce tryptophan, or overproduce tryptophan. For example, U.S. Patent No. 4,371,614 describes an *E. coli* strain transformed with a plasmid containing a wild type tryptophan operon.

Maximum titers of tryptophan disclosed in U.S. Patent No. 4,371,614 are about 230 ppm. Similarly, WO 8701130 describes an *E. coli* strain that has been genetically engineered to produce tryptophan and discusses increasing fermentative production of L-tryptophan. Those skilled in the art will recognize that organisms capable of producing tryptophan from glucose are also capable of utilizing other carbon and energy sources that can be converted to glucose or fructose-6-phosphate, with similar results. Exemplary carbon and energy sources include, but are not limited to, sucrose, fructose, starch, cellulose, or glycerol.

Tryptophan to Indole-3-pyruvate

Several polypeptides can be used to convert tryptophan to indole-3-pyruvate. Exemplary polypeptides include, without limitation, members of the enzyme classes (EC) 2.6.1.27, 1.4.1.19, 1.4.99.1, 2.6.1.28, 1.4.3.2, 1.4.3.3, 2.6.1.5, 2.6.1.-, 2.6.1.1, and 2.6.1.21. These classes include, without limitation, polypeptides termed tryptophan aminotransferase (also termed L-phenylalanine-2-oxoglutarate aminotransferase, tryptophan transaminase, 5-hydroxytryptophan-ketoglutaric transaminase, hydroxytryptophan aminotransferase, L-tryptophan aminotransferase, L-tryptophan transaminase, and L-tryptophan:2-oxoglutarate aminotransferase) which converts L-tryptophan and 2-oxoglutarate to indole-3-pyruvate and L-glutamate; D-tryptophan aminotransferase which converts D-tryptophan and a 2-oxo acid to indole-3-pyruvate and an amino acid; tryptophan dehydrogenase (also termed NAD(P)-L-tryptophan dehydrogenase, L-tryptophan dehydrogenase, L-Trp-dehydrogenase, TDH and L-tryptophan:NAD(P) oxidoreductase (deaminating)) which converts L-tryptophan and NAD(P) to indole-3-pyruvate and NH_3 and NAD(P)H; D-amino acid dehydrogenase, which converts D-amino acids and FAD to indole-3-pyruvate and NH_3 and $FADH_2$; tryptophan-phenylpyruvate transaminase (also termed L-tryptophan-α-ketoisocaproate aminotransferase and L-tryptophan:phenylpyruvate aminotransferase) which converts L-tryptophan and phenylpyruvate to indole-3-pyruvate and L-phenylalanine; L-amino acid oxidase (also termed ophio-amino-acid oxidase and L-amino-acid:oxygen oxidoreductase (deaminating)) which converts an L-amino acid and H_2O and O_2 to a 2-oxo acid and NH_3 and H_2O_2; D-amino acid oxidase (also termed ophio-amino-acid oxidase and D-amino-acid:oxygen oxidoreductase

(deaminating)) which converts a D-amino acid and H_2O and O_2 to a 2-oxo acid and NH_3 and H_2O_2; and tryptophan oxidase which converts L-tryptophan and H_2O and O_2 to indole-3-pyruvate and NH_3 and H_2O_2. These classes also contain tyrosine (aromatic) aminotransferase, aspartate aminotransferase, D-amino acid (or D-alanine) aminotransferase, and broad (multiple substrate) aminotransferase which have multiple aminotransferase activities, some of which can convert tryptophan and a 2-oxo acid to indole-3-pyruvate and an amino acid.

Eleven members of the aminotransferase class that have such activity are described below in Example 1, including a novel aminotransferase shown in SEQ ID NOS: 11 and 12. Therefore, this disclosure provides isolated nucleic acid and amino acid sequences having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or even at least 99% sequence identity to the sequences set forth in SEQ ID NOS: 11 and 12, respectively. Also encompassed by this disclosure are fragments and fusions of the sequences set forth in SEQ ID NOS: 11 and 12 that encode a polypeptide having aminotransferase activity or retaining aminotransferase activity. Exemplary fragments include, but are not limited to, at least 10, 12, 15, 20, 25, 50, 100, 200, 500, or 1000 contiguous nucleotides of SEQ ID NO: 11 or at least 6, 10, 15, 20, 25, 50, 75, 100, 200, 300 or 350 contiguous amino acids of SEQ ID NO: 12. The disclosed sequences (and variants, fragments, and fusions thereof) can be part of a vector. The vector can be used to transform host cells, thereby producing recombinant cells which can produce indole-3-pyruvate from tryptophan, and in some examples can further produce MP and/or monatin.

L-amino acid oxidases (1.4.3.2) are known, and sequences can be isolated from several different sources, such as *Vipera lebetine* (sp P81375), *Ophiophagus hannah* (sp P81383), *Agkistrodon rhodostoma* (spP81382), *Crotalus atrox* (sp P56742), *Burkholderia cepacia, Arabidopsis thaliana, Caulobacter cresentus, Chlamydomonas reinhardtii, Mus musculus, Pseudomonas syringae*, and *Rhodococcus str.* In addition, tryptophan oxidases are described in the literature and can be isolated, for example, from *Coprinus* sp. SF-1, Chinese cabbage with club root disease, *Arabidopsis thaliana*, and mammalian liver. One member of the L-amino acid oxidase class that can convert tryptophan to indole-3-pyruvate is discussed below in Example 3, as well as alternative sources for molecular cloning. Many D-amino acid oxidase genes are available in databases for molecular cloning.

Tryptophan dehydrogenases are known, and can be isolated, for example, from spinach, *Pisum sativum*, *Prosopis juliflora*, pea, mesquite, wheat, maize, tomato, tobacco, *Chromobacterium violaceum*, and *Lactobacilli*. Many D-amino acid dehydrogenase gene sequences are known.

As shown in FIGS. 11-13, if an amino acid oxidase, such as tryptophan oxidase, is used to convert tryptophan to indole-3-pyruvate, catalase can be added to reduce or even eliminate the presence of hydrogen peroxide.

Indole-3-lactate to Indole-3-pyruvate

The reaction that converts indole-3-lactate to indole-3-pyruvate can be catalyzed by a variety of polypeptides, such as members of the 1.1.1.110, 1.1.1.27, 1.1.1.28, 1.1.2.3, 1.1.1.222, 1.1.1.237, 1.1.3.-, or 1.1.1.111 classes of polypeptides. The 1.1.1.110 class of polypeptides includes indolelactate dehydrogenases (also termed indolelactic acid: NAD^+ oxidoreductase). The 1.1.1.27, 1.1.1.28, and 1.1.2.3 classes include lactate dehydrogenases (also termed lactic acid dehydrogenases, lactate: NAD^+ oxidoreductase). The 1.1.1.222 class contains (R)-4-hydroxyphenyllactate dehydrogenase (also termed D-aromatic lactate dehydrogenase, R-aromatic lactate dehydrogenase, and R-3-(4-hydroxyphenyl)lactate:$NAD(P)^+$ 2-oxidoreductase) and the 1.1.1.237 class contains 3-(4-hydroxyphenylpyruvate) reductase (also termed hydroxyphenylpyruvate reductase and 4-hydroxyphenyllactate: NAD^+ oxidoreductase). The 1.1.3.- class contains lactate oxidases, and the 1.1.1.111 class contains (3-imidazol-5-yl) lactate dehydrogenases (also termed (S)-3-(imidazol-5-yl)lactate:$NAD(P)^+$ oxidoreductase). It is likely that several of the polypeptides in these classes allow for the production of indole-3-pyruvate from indole-3-lactic acid. Examples of this conversion are provided in Example 2.

Chemical reactions can also be used to convert indole-3-lactic acid to indole-3-pyruvate. Such chemical reactions include an oxidation step that can be accomplished using several methods, for example: air oxidation using a B2 catalyst (China Chemical Reporter, vol. 13, no. 28, pg. 18(1), 2002), dilute permanganate and perchlorate, or hydrogen peroxide in the presence of metal catalysts.

Indole-3-pyruvate to 2-hydroxy 2-(indol-3ylmethyl)-4-keto glutaric acid (MP)

Several known polypeptides can be used to convert indole-3-pyruvate to MP. Exemplary polypeptide classes include 4.1.3.-, 4.1.3.16, 4.1.3.17, and 4.1.2.-. These classes include carbon-carbon synthases/lyases, such as aldolases that catalyze the condensation of two carboxylic acid substrates. Polypeptide class EC 4.1.3.- are synthases/lyases that form carbon-carbon bonds utilizing oxo-acid substrates (such as indole-3-pyruvate) as the electrophile, while EC 4.1.2.- are synthases/lyases that form carbon-carbon bonds utilizing aldehyde substrates (such as benzaldehyde) as the electrophile.

For example, the polypeptide described in EP 1045-029 (EC 4.1.3.16, 4-hydroxy-2-oxoglutarate glyoxylate-lyase also termed 4-hydroxy-2-oxoglutarate aldolase, 2-oxo-4-hydroxyglutarate aldolase or KHG aldolase) converts glyoxylic acid and pyruvate to 4-hydroxy-2-ketoglutaric acid, and the polypeptide 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17, also termed 4-hydroxy-4-methyl-2-oxoglutarate pyruvate-lyase or ProA aldolase), condenses two keto-acids such as two pyruvates to 4-hydroxy-4-methyl-2-oxoglutarate. Reactions utilizing these lyases are described herein.

FIGS. 1-2 and 11-13 show schematic diagrams of these reactions in which a 3-carbon (C3) molecule is combined with indole-3-pyruvate. Many members of EC 4.1.2.- and 4.1.3.-, particularly PLP-utilizing polypeptides, can utilize C3 molecules that are amino acids such as serine, cysteine, and alanine, or derivatives thereof. Aldol condensations catalyzed by representatives of EC 4.1.2.- and 4.1.3.- require the three carbon molecule of this pathway to be pyruvate or a derivative of pyruvate. However, other compounds can serve as a C3 carbon source and be converted to pyruvate. Alanine can be transaminated by many PLP-utilizing transaminases, including many of those mentioned above, to yield pyruvate. Pyruvate and ammonia can be obtained by beta-elimination reactions (such as those catalyzed by tryptophanase or β-tyrosinase) of L-serine, L-cysteine, and derivatives of serine and cysteine with sufficient leaving groups, such as O-methyl-L-serine, O-benzyl-L-serine, S-methylcysteine, S-benzylcysteine, S-alkyl-L-cysteine, O-acyl-L-serine, and 3-chloro-L-alanine. Aspartate can serve as a source of pyruvate in PLP-mediated beta-lyase reactions such as those catalyzed by tryptophanase (EC 4.1.99.1) and/or β-tyrosinase (EC 4.1.99.2, also termed tyrosine-phenol lyase). The rate of beta-lyase reactions can be increased by performing site-directed mutagenesis on the (4.1.99.1-2) polypeptides as described by

Mouratou *et al.* (*J. Biol. Chem* 274:1320-5, 1999) and in Example 8. These modifications allow the polypeptides to accept dicarboxylic amino acid substrates. Lactate can also serve as a source of pyruvate, and is oxidized to pyruvate by the addition of lactate dehydrogenase and an oxidized cofactor or lactate oxidase and oxygen. Examples of these reactions are described below. For example, as shown in FIG. 2 and FIGS. 11-13, ProA aldolase can be contacted with indole-3-pyruvate when pyruvate is used as the C3 molecule.

The MP can also be generated using chemical reactions, such as the aldol condensations provided in Example 5.

MP to Monatin

Conversion of MP to monatin can be catalyzed by one or more of: tryptophan aminotransferases (2.6.1.27), tryptophan dehydrogenases (1.4.1.19), D-amino acid dehydrogenases (1.4.99.1), glutamate dehydrogenases (1.4.1.2-4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminases (2.6.1.28), or more generally members of the aminotransferase family (2.6.1.-) such as aspartate aminotransferase (EC 2.6.1.1), tyrosine (aromatic) aminotransferase (2.6.1.5), D-tryptophan aminotransferase, or D-alanine (2.6.1.21) aminotransferase (FIG. 2). Eleven members of the aminotransferase class are described below (Example 1), including a novel member of the class shown in SEQ ID NOS: 11 and 12, and reactions demonstrating the activity of aminotransferase and dehydrogenase enzymes are provided in Example 7.

This reaction can also be performed using chemical reactions. Amination of the keto acid (MP) is performed by reductive amination using ammonia and sodium cyanoborohydride.

FIGS. 11-13 show additional polypeptides that can be used to convert MP to monatin, as well as providing increased yields of monatin from indole-3-pyruvate or tryptophan. For example, if aspartate is used as the amino donor, aspartate aminotransferase can be used to convert the aspartate to oxaloacetate (FIG. 11). The oxaloacetate is converted to pyruvate and carbon dioxide by a decarboxylase, such as oxaloacetate decarboxylase (FIG. 11). In addition, if lysine is used as the amino donor, lysine epsilon aminotransferase can be used to convert the lysine to allysine (FIG. 12). The allysine is spontaneously converted to 1-piperideine 6-carboxylate (FIG. 12). If a polypeptide capable of catalyzing reductive amination reactions

(e.g., glutamate dehydrogenase) is used to convert MP to monatin, a polypeptide that can recycle NAD(P)H and/or produce a volatile product (FIG. 13) can be used, such as formate dehydrogenase.

Additional Considerations in the Design of the Biosynthetic Pathways

Depending on which polypeptides are used to generate indole-3-pyruvate, MP, and/or monatin, cofactors, substrates, and/or additional polypeptides can be provided to the production cell to enhance product formation. In addition, genetic modification can be designed to enhance production of products such as indole-3-pyruvate, MP, and/or monatin. Similarly, a host cell used for monatin production can be optimized.

Removal of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a product that, if generated, can be damaging to production cells, polypeptides or products (e.g., intermediates) produced. The L-amino acid oxidase described above generates H_2O_2 as a product. Therefore, if L-amino acid oxidase is used, the resulting H_2O_2 can be removed or its levels decreased to reduce potential injury to the cell or product.

Catalases can be used to reduce the level of H_2O_2 in the cell (FIGS. 11-13). The production cell can express a gene or cDNA sequence that encodes a catalase (EC 1.11.1.6), which catalyzes the decomposition of hydrogen peroxide into water and oxygen gas. For example, a catalase can be expressed from a vector transfected into the production cell. Examples of catalases that can be used include, but are not limited to: tr|Q9EV50 (*Staphylococcus xylosus*), tr|Q9KBE8 (*Bacillus halodurans*), tr|Q9URJ7 (*Candida albicans*), tr|P77948 (*Streptomyces coelicolor*), tr|Q9RBJ5 (*Xanthomonas campestris*) (SwissProt Accession Nos.). Biocatalytic reactors utilizing L-amino acid oxidase, D-amino acid oxidase, or tryptophan oxidase can also contain a catalase polypeptide.

Modulation of pyridoxal-5'-phosphate (PLP) Availability

As shown in FIG. 1, PLP can be utilized in one or more of the biosynthetic steps described herein. The concentration of PLP can be supplemented so that PLP does not become a limitation on the overall efficiency of the reaction.

The biosynthetic pathway for vitamin B_6 (the precursor of PLP) has been thoroughly studied in *E. coli*, and some of the proteins have been crystallized (Laber *et al.*, *FEBS Letters*, 449:45-8, 1999). Two of the genes (*epd* or *gapB* and *serC*) are required in other metabolic pathways, while three genes (*pdxA*, *pdxB*, and *pdxJ*) are unique to pyridoxal phosphate biosynthesis. One of the starting materials in the *E. coli* pathway is 1-deoxy-D-xylulose-5-phosphate (DXP). Synthesis of this precursor from common 2 and 3 carbon central metabolites is catalyzed by the polypeptide 1-deoxy-D-xylulose-5-phosphate synthase (DXS). The other precursor is a threonine derivative formed from the 4-carbon sugar, D-erythrose 4-phosphate. The genes required for the conversion to phospho-4-hydroxyl-L threonine (HTP) are *epd*, *pdxB*, and *serC*. The last reaction for the formation of PLP is a complex intramolecular condensation and ring-closure reaction between DXP and HTP, catalyzed by the gene products of *pdxA* and *pdxJ*.

If PLP becomes a limiting nutrient during the fermentation to produce monatin, increased expression of one or more of the pathway genes in a production host cell can be used to increase the yield of monatin. A host organism can contain multiple copies of its native pathway genes or copies of non-native pathway genes can be incorporated into the organism's genome. Additionally, multiple copies of the salvage pathway genes can be cloned into the host organism.

One salvage pathway that is conserved in all organisms recycles the various derivatives of vitamin B_6 to the active PLP form. The polypeptides involved in this pathway are *pdxK* kinase, *pdxH* oxidase, and *pdxY* kinase. Over-expression of one or more of these genes can increase PLP availability.

Vitamin B_6 levels can be elevated by elimination or repression of the metabolic regulation of the native biosynthetic pathway genes in the host organism. PLP represses polypeptides involved in the biosynthesis of the precursor threonine derivative in the bacterium *Flavobacterium sp.* strain 238-7. This bacterial strain, freed of metabolic control, overproduces pyridoxal derivatives and can excrete up to 20 mg/L of PLP. Genetic manipulation of the host organism producing monatin in a similar fashion will allow the increased production PLP without over-expression of the biosynthetic pathway genes.

Ammonium Utilization

Tryptophanase reactions can be driven toward the synthetic direction (production of tryptophan from indole) by making ammonia more available or by removal of water. Reductive amination reactions, such as those catalyzed by glutamate dehydrogenase, can also be driven forward by an excess of ammonium.

Ammonia can be made available as an ammonium carbonate or ammonium phosphate salt in a carbonate or phosphate buffered system. Ammonia can also be provided as ammonium pyruvate or ammonium formate. Alternatively, ammonia can be supplied if the reaction is coupled with a reaction that generates ammonia, such as glutamate dehydrogenase or tryptophan dehydrogenase. Ammonia can be generated by addition of the natural substrates of EC 4.1.99.- (tyrosine or tryptophan), which will be hydrolyzed to phenol or indole, pyruvate and NH_3. This also allows for an increased yield of synthetic product over the normal equilibrium amount by allowing the enzyme to hydrolyze its preferred substrate.

Removal of products and byproducts

The conversion of tryptophan to indole-3-pyruvate via a tryptophan aminotransferase can adversely affect the production rate of indole-3-pyruvate because the reaction produces glutamate and requires the co-substrate 2-oxoglutarate (α-ketoglutarate). Glutamate can cause inhibition of the aminotransferase, and the reaction can consume large amounts of the co-substrate. Moreover, high glutamate concentrations can be detrimental to downstream separation processes.

The polypeptide glutamate dehydrogenase (GLDH) converts glutamate to 2-oxoglutarate, thereby recycling the co-substrate in the reaction catalyzed by tryptophan aminotransferase. GLDH also generates reducing equivalents (NADH or NADPH) that can be used to generate energy for the cell (ATP) under aerobic conditions. The utilization of glutamate by GLDH also reduces byproduct formation. Additionally, the reaction generates ammonia, which can serve as a nitrogen source for the cell or as a substrate in a reductive amination for the final step shown in FIG. 1. Therefore, a production cell that over-expresses a GLDH polypeptide can be used to increase the yield and reduce the cost of media and/or separation processes.

In the tryptophan to monatin pathway, the amino donor of step three (e.g., glutamate or aspartate) can be converted back to the amino acceptor required for step 1 (e.g., 2-oxo-glutarate or oxaloacetate), if an aminotransferase from the appropriate enzyme classes is used. Utilization of two separate transaminases for this pathway, in which the substrate of one transaminase does not competitively inhibit the activity of the other transaminase, can increase the efficiency of this pathway.

Many of the reactions in the described pathways are reversible and can, therefore, reach an equilibrium between substrates and products. The yield of the pathway can be increased by continuous removal of the products from the polypeptides. For example, secretion of monatin into the fermentation broth using a permease or other transport protein, or selective crystallization of monatin from a biocatalytic reactor stream with concomitant recycle of substrates will increase the reaction yield.

Removal of byproducts via additional enzymatic reactions or via substitution of amino donor groups is another way to increase the reaction yield. Several examples are discussed in Example 13 and shown in FIGS. 11-13. For example, a byproduct can be produced that is unavailable to react in the reverse direction, either by phase change (evaporation) or by spontaneous conversion to an unreactive end product, such as carbon dioxide.

Modulation of the Substrate Pools

The indole pool can be modulated by increasing production of tryptophan precursors and/or altering catabolic pathways involving indole-3-pyruvate and/or tryptophan. For example, the production of indole-3-acetic acid from indole-3-pyruvate can be reduced or eliminated by functionally deleting the gene coding for EC 4.1.1.74 in the host cell. Production of indole from tryptophan can be reduced or eliminated by functionally deleting the gene coding for EC 4.1.99.1 in the host cell. Alternatively, an excess of indole can be utilized as a substrate in an *in vitro* or *in vivo* process in combination with increased amounts of the gene coding for EC 4.1.99.1 (Kawasaki *et al.*, *J. Ferm. and Bioeng.*, 82:604-6, 1996). In addition, genetic modifications can be made to increase the level of intermediates such as D-erythrose-4-phosphate and chorismate.

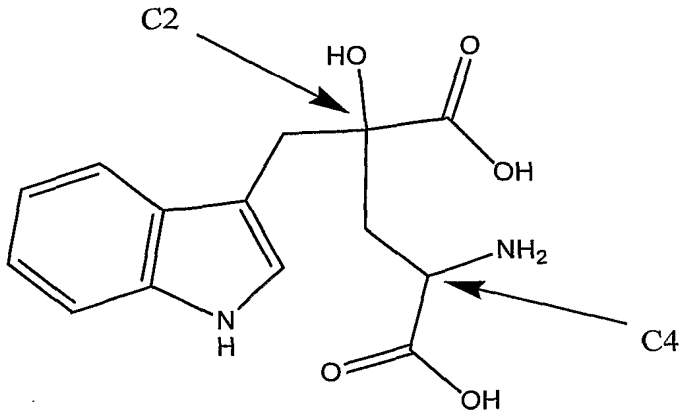
Tryptophan production is regulated in most organisms. One mechanism is via feedback inhibition of certain enzymes in the pathway; as tryptophan levels increase, the production rate of tryptophan decreases. Thus, when using a host cell engineered to produce monatin via a tryptophan intermediate, an organism can be used that is not sensitive to tryptophan concentrations. For example, a strain of *Catharanthus roseus* that is resistant to growth inhibition by various tryptophan analogs was selected by repeated exposure to high concentrations of 5-methyltryptophan (Schallenberg and Berlin, Z Naturforsch 34:541-5, 1979). The resulting tryptophan synthase activity of the strain was less effected by product inhibition, likely due to mutations in the gene. Similarly, a host cell used for monatin production can be optimized.

Tryptophan production can be optimized through the use of directed evolution to evolve polypeptides that are less sensitive to product inhibition. For example, screening can be performed on plates containing no tryptophan in the medium, but with high levels of non-metabolizable tryptophan analogs. U.S. Pat. Nos. 5,756,345; 4,742,007; and 4,371,614 describe methods used to increase tryptophan productivity in a fermentation organism. The last step of tryptophan biosynthesis is the addition of serine to indole; therefore the availability of serine can be increased to increase tryptophan production.

The amount of monatin produced by a fermentation organism can be increased by increasing the amount of pyruvate produced by the host organism. Certain yeasts, such as *Trichosporon cutaneum* (Wang et al., Lett. Appl. Microbiol. 35:338-42, 2002) and *Torulopsis glabrata* (Li et al., Appl Microbiol. Biotechnol. 57:451-9, 2001) overproduce pyruvate and can be used to practice the methods disclosed herein. In addition, genetic modifications can be made to organisms to promote pyruvic acid production, such as those in *E. coli* strain W1485*lip2* (Kawasaki et al., J. Ferm. and Bioeng. 82:604-6, 1996).

Controlling Chirality

The taste profile of monatin can be altered by controlling its stereochemistry (chirality). For example, different monatin stereoisomers may be desired in different blends of concentrations for different food systems. Chirality can be controlled via a combination of pH and polypeptides.



Racemization at the C-4 position of monatin (see numbered molecule above) can occur by deprotonation and reprotonation of the alpha carbon, which can occur by a shift in pH or by reaction with the cofactor PLP bound to an enzyme such as a racemase or free in solution. In a microorganism, the pH is unlikely to shift enough to cause the racemization, but PLP is abundant. Methods to control the chirality with polypeptides depend upon the biosynthetic route utilized for monatin production.

When monatin is formed using the pathway shown in FIG. 2, the following can be considered. In a biocatalytic reaction, the chirality of carbon-2 can be determined by an enzyme that converts indole-3-pyruvate to MP. Multiple enzymes (e.g., from EC 4.1.2.-, 4.1.3.-) can convert indole-3-pyruvate to MP, thus, the enzyme that forms the desired stereoisomer can be chosen. Alternatively, the enantiospecificity of the enzyme that converts indole-3-pyruvate to MP can be modified through the use of directed evolution, or catalytic antibodies can be engineered to catalyze the desired reaction. Once MP is produced (either enzymatically or by chemical condensation), the amino group can be added stereospecifically using a transaminase, such as those described herein. Either the R or S configuration of carbon-4 can be generated depending on whether a D- or L- aromatic acid aminotransferase is used. Most aminotransferases are specific for the L-stereoisomer; however, D-tryptophan aminotransferases exist in certain plants (Kohiba and Mito, Proceedings of the 8th International Symposium on Vitamin B_6 and Carbonyl Catalysis, Osaka, Japan 1990). Moreover, D-alanine aminotransferases (2.6.1.21), D-methionine-pyruvate aminotransferases (2.6.1.41), and both (R)-3-amino-2-methylpropanoate aminotransferase (2.6.1.61) and (S)-3-amino-2-methylpropanoate aminotransferase (2.6.1.22) have been identified. Certain aminotransferases may only accept the substrate for this reaction with a particular

configuration at the C2 carbon. Therefore, even if the conversion to MP is not stereospecific, the stereochemistry of the final product can be controlled through the appropriate selection of a transaminase. Since the reactions are reversible, the unreacted MP (undesired stereoisomer) can be recycled back to its constituents, and a racemic mixture of MP can be reformed.

Activating Substrates

Phosphorylated substrates, such as phosphoenolpyruvate (PEP), can be used in the reactions disclosed herein. Phosphorylated substrates can be more energetically favorable and, therefore, can be used to increase the reaction rates and/or yields. In aldol condensations, the addition of a phosphate group stabilizes the enol tautomer of the nucleophilic substrate, making it more reactive. In other reactions, a phosphorylated substrate can provide a better leaving group. Similarly, substrates can be activated by conversion to CoA derivatives or pyrophosphate derivatives.

Use of monatin in a beverage composition

The S,S stereoisomer of monatin is approximately 50-200 times sweeter than sucrose by weight. The R,R stereoisomer of monatin is approximately 2000-2400 times sweeter than sucrose by weight. The sweetness of the monatin is calculated using experienced sensory evaluators in a sweetness comparison procedure, where a test sweetener solution is matched for sweetness intensity against one of a series of reference solutions. The solutions may be prepared, for example, using a buffer comprising 0.16% (w/v) citric acid and 0.02% (w/v) sodium citrate at ~pH 3.0.

Specifically, one may assess sweetness of a sweetener relative to sucrose by using a panel of trained sensory evaluators experienced in the sweetness estimation procedure. All samples (in same buffers) are served in duplicate at a temperature of 22°C ± 1°C. Sample solutions may be prepared, for example, using a buffer comprising 0.16% (w/v) citric acid and 0.02% (w/v) sodium citrate at ~pH 3.0. Test solutions, coded with 3 digit random number codes, are presented individually to panelists, in random order. Sucrose reference standards, ranging from 2.0 – 10.0% (w/v) sucrose, increasing in steps of 0.5% (w/v) sucrose are also provided. Panelists are asked to estimate sweetness by comparing the sweetness of the test solution to the sucrose standards. This is carried out by taking 3 sips of the test solution, followed by a

sip of water, followed by 3 sips of sucrose standard followed by a sip of water, etc. Panelists estimate the sweetness to one decimal place, e.g., 6.8, 8.5. A five minute rest period is imposed between evaluating the test solutions. Panelists are also asked to rinse well and eat a cracker to reduce any potential carry over effects.

Sucrose equivalent value (SEV) (e.g., % sucrose), determined by the panel of trained sensory evaluators, is plotted as a function of monatin concentration to obtain a dose response curve. A polynomial curve fit is applied to the dose response curve and used to calculate the sweetness intensity or potency at a particular point, e.g., 8% SEV, by dividing the sucrose equivalent value (SEV) by the monatin concentration (e.g., % monatin). *See e.g.*, FIG. 15 (R,R/S,S monatin dose response curve); FIG. 14 (R,R monatin dose response curve). The above-mentioned sweetness intensities for S,S and R,R monatin (i.e., approximately 50-200 times sweeter and approximately 2000-2400 times sweeter than sucrose by weight, respectively) were determined at approximately 8% SEV.

Monatin is soluble in aqueous solutions in concentrations that are appropriate for consumption. Various blends of monatin stereoisomers may be qualitatively better in certain matrices, or in blending with other sweeteners. Blends of monatin with other sweeteners may be used to maximize the sweetness intensity and/or profile, and minimize cost. Monatin may be used in combination with other sweeteners and/or other ingredients to generate a temporal profile similar to sucrose, or for other benefits.

For example, monatin may be blended with other nutritive and nonnutritive sweeteners to achieve particular flavor profiles or calorie targets. Thus, sweetener compositions can include combinations of monatin with one or more of the following sweetener types: (1) sugar alcohols (such as erythritol, sorbitol, maltitol, mannitol, lactitol, xylitol, isomalt, low glycemic syrups, etc.); (2) other high intensity sweeteners (such as aspartame, sucralose, saccharin, acesulfame-K, stevioside, cyclamate, neotame, thaumatin, alitame, dihydrochalcone, monellin, glycyrrihizin, mogroside, phyllodulcin, mabinlin, brazzein, circulin, pentadin, etc.) and (3) nutritive sweeteners (such as sucrose, D-tagatose, invert sugar, fructose, corn syrup, high fructose corn syrup (HFCS), glucose/dextrose, trehalose, isomaltulose, etc.). Monatin may be used in such blends as a taste modifier to suppress aftertaste, enhance other flavors such as lemon, or improve the temporal flavor profile. Data

also indicate that monatin is quantitatively synergistic with cyclamates (which are used in Europe), but no significant quantitative synergy was noted with aspartame, saccharin, acesulfame-K, sucralose, or carbohydrate sweeteners.

Because monatin is not a carbohydrate, monatin can be used to lower the carbohydrate content in beverage compositions. In one embodiment, an amount of a beverage composition comprising monatin contains less calories and carbohydrates than the same amount of a beverage composition containing sugar (e.g., sucrose and/or high fructose corn syrup) in place of the monatin. In other embodiments, beverage compositions comprising monatin (e.g., comprising monatin and one or more carbohydrates) provide a mouthfeel, flavor and sweetness over time that is comparable to that provided by similar beverage compositions containing only carbohydrates as the sweetener.

Monatin is stable in a dry form, and has a desirable taste profile alone or when mixed with carbohydrates. It does not appear to irreversibly break down, but rather forms lactones and/or lactams at low pHs (in aqueous buffers) and reaches an equilibrium. It can racemize at the 4 position slowly over time in solution, but typically this occurs at high pHs. In general, the stability of monatin is comparable to or better than aspartame and the taste profile of monatin is comparable to or better than other quality sweeteners, such aspartame, alitame, and sucralose. Monatin does not have the undesirable aftertaste associated with some other high intensity sweeteners such as saccharin and stevioside.

In some embodiments, beverage compositions comprising monatin also include one or more of the following: buffers, bulking agents, thickeners, fats, flavorings, coloring agents (also called colorants or colors), sweeteners and flow agents. Beverage compositions can be formulated to have a particular sweetness profile, e.g., by tailoring the amount of monatin or other sweeteners present in the beverage or by tailoring the amount or type of other additives, including flavoring agents or acids, present in the composition. In other embodiments, all ingredients used in beverage compositions are food grade and generally recognized as safe.

In some embodiments, beverage compositions comprising monatin further comprise food grade antioxidants. Examples of such antioxidants include vitamin C (e.g., ascorbic acid, magnesium ascorbyl phosphate), erythorbate (isoascorbic acid), carotenoids such as lutein,

lycopene and beta-carotene, tocopherols (e.g., α-tocopherol (natural vitamin E), γ-tocopherol, δ- tocopherol), hydroxycinnamates (e.g., neochlorogenic acid and chlorogenic acid), glutathione, phenolics (e.g., cocoa phenols, red wine phenols, phenolics in prunes), butylated hyroxyanisole (BHA), butylated hydroxytolulene (BHT), tertiary butylhydroquinone (TBHQ), propyl gallate, nisin, green tea extract and rosemary extract. In other embodiments, beverage compositions comprising monatin further comprise certain preservatives, such as sodium benzoate and/or potassium sorbate.

In other embodiments, beverage compositions comprising monatin further comprise one or more ingredients that prevent non-enzymatic browning reactions (e.g., browning due to Maillard reactions). Such ingredients may include, but are not limited to, sulfites and sulfiting agents (e.g., sulfur dioxide, sodium sulfite, sodium or potassium bisulfite, metabisulfites, sulfhydryl-containing amino acids), calcium chloride and other inorganic halides, antioxidants, and compounds that affect the water activity (e.g., glycerol, sorbitol and trehalose).

In some embodiments, monatin-containing beverage concentrates such as dry beverage mixes can be readily dispersed to prepare chocolate beverages, fruit beverages, malted beverages, or lemonade. In other embodiments, a beverage concentrate is a beverage syrup that can be used to prepare carbonated soft drinks. A carbonated beverage can be prepared, for example, by diluting a beverage syrup containing water, monatin, and flavorings, with carbonated water. In some embodiments, the beverage syrup also contains other sweeteners and/or additives. Beverage syrups can be prepared, for example, by mixing all of the ingredients and heating to solubilize. Beverage syrups may include, for example, at least 80% water (e.g., at least 85%, 90%, or 95% water).

In certain embodiments, monatin is present in an amount that ranges from about 0.0003 to about 1 % of the beverage composition (i.e., about 3 to about 10,000 ppm) (e.g., about 0.0005 to about 0.2 %), including any particular value within that range (e.g., 0.0003 %, 0.005 %, 0.06 % or 0.2 % of the beverage composition). For example, a beverage composition may comprise 0.0005 to 0.005 % (e.g., 0.001 to 0.0045 %) of the R,R monatin, or 0.005 to 0.2 % (e.g., 0.01 to 0.175 %) of S,S monatin.

One of skill in the art will recognize that combinations of sweeteners can be used to provide the desired taste and caloric count of a beverage composition. Thus, the amount of sweetener in a beverage composition depends upon the choice of sweeteners and desired sweetness intensity. Sweeteners are commercially available, e.g., through Cargill Inc. (Wayzata, MN) and McNeil Specialty (Fort Washington, PA). In one embodiment, a beverage composition includes a blend of monatin and a sweetener (e.g., sucrose or high fructose corn syrup). For example, a beverage composition can include monatin and a bulk sweetener.

Bulk sweeteners may be chosen from, for example, sugar sweeteners, sugarless sweeteners, lower glycemic carbohydrates, and a combination thereof. Sugar sweeteners can include, for example, a corn sweetener, sucrose, dextrose (e.g., Cerelose dextrose), maltose, dextrin, maltodextrin, invert sugar, fructose, high fructose corn syrup, levulose, galactose, corn syrup solids, galactose, trehalose, isomaltulose, fructo-oligosaccharides (such as kestose or nystose), higher molecular weight fructo-oligosaccharides or a combination thereof. High fructose corn syrup (HFCS) and other corn derived sweeteners, for example, are combinations of dextrose (glucose) and fructose. In addition, sugar sweeteners include fruit sugars, maple syrup, and honey, or combinations thereof. In one embodiment, 0.0003 to 0.15 % monatin (e.g., 0.0006 to 0.004 % of R,R monatin) and 2 to 10 % (e.g., 3 to 10% or 4 to 6%) of sucrose or high fructose corn syrup can be used in a beverage composition.

In another embodiment, a beverage composition includes a sugarless sweetener and/or a lower glycemic carbohydrate (i.e., one with a lower glycemic index than glucose). Sugarless sweeteners or lower glycemic carbohydrates include, but are not limited to, D-tagatose, sorbitol (including amorphous and crystalline sorbitol), mannitol, xylitol, lactitol, erythritol, maltitol, hydrogenated starch hydrolysates, isomalt, D-psicose, 1,5 anhydro D-fructose or a combination thereof.

In certain embodiments, beverage compositions comprising monatin also comprise high intensity sweeteners. In some embodiments, high intensity sweeteners are at least 20 times sweeter than sucrose (i.e., 20 X sucrose). Such high intensity sweeteners include, but are not limited to, sucralose, aspartame, saccharin and its salts, salts of acesulfame (e.g., acesulfame K), alitame, thaumatin, dihydrochalcones (e.g., neohesperidin dihydrochalcone), neotame, cyclamic acid and its salts (i.e., cyclamates), stevioside (extracted from leaves of *Stevia*

rebaudiana), mogroside (extracted from Lo Han Guo fruit), glycyrrhizin, phyllodulcin (extracted from leaves of *Hydrangea macrophylla*, about 400 to 600 X sucrose), monellin, mabinlin, brazzein, circulin, pentadin, either alone or in combination.

Sweetness enhancers, which only are sweet in the presence of other compounds such as acids, also can be used in a beverage composition. Non-limiting examples of sweetness enhancers (also known as sweetness potentiators) include curculin, miraculin, cynarin, chlorogenic acid, caffeic acid, strogins, arabinogalactan, maltol and dihyroxybenzoic acids. In certain embodiments, beverage compositions comprising monatin also include flavor enhancers or stabilizers, such as Sucramask™ or trehalose.

Food grade natural or artificial colorants may optionally be included in the beverage compositions. These colorants may be selected from those generally known and available in the art, including synthetic colors (e.g., azo dyes, triphenylmethanes, xanthenes, quinines, and indigoids), caramel color, titanium dioxide, red #3, red #40, blue #1, and yellow #5. Natural coloring agents such as beet juice (beet red), carmine, curcumin, lutein, carrot juice, berry juices, spice extractives (turmeric, annatto and/or paprika), and carotenoids, for example, may also be used. The type and amount of colorant selected will depend on the end product and consumer preference.

In some embodiments, beverage compositions also include one or more natural or synthetic flavorings. Suitable flavorings include citrus and non-citrus fruit flavors; spices; herbs; botanicals; chocolate, cocoa, or chocolate liquor; coffee; flavorings obtained from vanilla beans; nut extracts; liqueurs and liqueur extracts; fruit brandy distillates; aromatic chemicals, imitation flavors; and concentrates, extracts, or essences of any of the same. Citrus flavors include, for example, lemon, lime, orange, tangerine, grapefruit, citron or kumquat. Many flavorings are available commercially from, e.g., Rhodia USA (Cranbury, NJ); IFF (South Brunswick, NJ); Wild Flavors, Inc. (Erlanger, KY); Silesia Flavors, Inc. (Hoffman Estates, IL), Chr. Hansen (Milkwaukee, WI), and Firmenisch (Princeton, NJ).

For example, a beverage syrup for preparing a carbonated soft drink can include a natural cola flavor (e.g., from Kola nut extract) that can be used to impart a cola flavor to the beverage. In some embodiments, flavorings can be formed into an emulsion, which is then

dispersed into the beverage syrup. Emulsion droplets usually have a specific gravity less than that of the water and therefore can form a separate phase. Weighting agents, emulsifiers, and emulsion stabilizers can be used to stabilize the flavor emulsion droplets. Examples of such emulsifiers and emulsion stabilizer include gums, pectins, cellulose, polysorbates, sorbitan esters and propylene glycol alginates. In some embodiments, cola flavor emulsions represent 0.8 to 1.5 % of a beverage syrup. In other embodiments, additional flavorings that can be used to enhance the cola flavor include citrus flavors, such as lemon, lime, orange, tangerine, grapefruit, citron or kumquat, and spice flavors such as clove and vanilla. In other embodiments, citrus flavors (e.g., natural lemon or lime flavor) represent about 0.03 to 0.06 % of a beverage syrup and spice flavors (e.g., vanilla) represent 0.5 to 1.5% of a beverage syrup.

The pH of a beverage syrup can be controlled by the addition of acids (e.g., inorganic or organic acids). Typically, the pH of the beverage syrup ranges from 2.5 to about 5 (e.g., 2.5 to about 4.0). A particularly useful inorganic acid includes phosphoric acid, which can be present in its undissociated form, or as an alkali metal salt (e.g., potassium or sodium hydrogen phosphate, or potassium or sodium dihydrogen phosphate salts). Non-limiting examples of organic acids that can be used include citric acid, malic acid, fumaric acid, adipic acid, gluconic acid, glucuronolactone, hydroxycitric acid, tartaric acid, ascorbic acid, acetic acid or mixtures thereof. These acids can be present in their undissociated form or as their respective salts.

In some embodiments, the beverage syrup further comprise caffeine (e.g., from the natural cola flavor). Caffeine also can be added separately.

In one embodiment, a carbonated beverage may be prepared by diluting a beverage syrup with carbonated water such that the resulting beverage contains 15 to 25 % of the syrup and 75 to 85% water. Alternatively, non-carbonated water can be used to dilute the syrup to prepare the beverage then carbon dioxide can be introduced into the beverage to achieve carbonation. In another embodiment, the carbonated beverage typically is placed into a container such as a bottle or can and then sealed. Any conventional carbonation methodology can be used to make the carbonated beverages of this invention.

In some embodiments, the beverage compositions can be dried beverage mixes. It is noted that "dry" material may contain residual levels of liquid. For instance, a beverage mix can be a malted beverage mix, chocolate-flavored beverage mix, or a powdered fruit drink mix such as Kool-Aid® or Crystal Light®. In one embodiment, dried beverage mixes can be prepared by wet-mixing liquid ingredients in solution and vacuum drying the ingredients to provide a dry cake, followed by pulverizing the dry cake to a base powder. Ingredients such as oil, emulsifiers, and water can be used to blend in further dry ingredients, such as adding a cocoa powder to the base powder.

In another embodiment, a base beverage powder that does not typically have a sweetener, such as a lemonade packet, which is typically combined with sucrose by the consumer, can be blended with a high intensity sweetener such as monatin. The blending can be facilitated, for example, by using a diluent or bulking agent such as maltodextrin, hydrolyzed starch, dextrose, polydextrin, and inulin.

In other embodiments, malted beverage mixes include dry beverage ingredients, such as, for example, a powdered protein source such as milk powder, skim milk powder, egg protein powder, vegetable or grain protein isolates such as soy protein isolates, malt powders, hydrolysed cereal powders, starch powders, other carbohydrate powders, vitamins, minerals, cocoa powders, and powdered flavoring agents, or any combination of such ingredients. Liquid malted beverage ingredients can include, for example, one or more of fats and oils, liquid malt extracts, liquid sweeteners such as honey and glucose syrup, and liquid protein sources such as vegetable protein concentrates, or any combination thereof. Suitable fats include, without limitation, partially or fully hydrogenated vegetable oils such as cotton seed oil, soybean oil, corn oil, sunflower oil, palm oil, canola oil, palm kernel oil, peanut oil, rice oil, safflower oil, coconut oil, rape seed oil, and their mid- and high-oleic counterparts; or any combination thereof. Animal fats such as butter fat also can be used. The amount of each malted beverage ingredient can vary depending on the desired formulation. In some embodiments, monatin can be combined with a bulk sweetener as discussed above.

In some embodiments, fruit beverage premixes include citric acid (e.g., 60 to 70 %), flavorings (e.g., 2 to 4 %), colorants (e.g., 0.001 to 1 %), monatin, calcium phosphate (e.g., 0 to 25 %), a clouding agent (e.g., 0 to 5%), and ascorbic acid (e.g., 0 to 2%). For example, a

fruit beverage mix may include 64.9% citric acid, 20.5% calcium phosphate, 3.9% of a clouding agent, 0.78 ascorbic acid, 2.7% flavors, 0.1% colors, and monatin. In some embodiments, monatin can be combined with a bulk sweetener as discussed above. In another embodiment, to prepare a fruit beverage, the premix can be reconstituted with water such that the resulting beverage contains about 0.5 to 1.5 % (e.g., 0.75%) of the mix.

In one embodiment, a dry chocolate drink composition can include skimmed milk powder (e.g., about 20 to 30%), whey powder (e.g., 35 to 45%), coffee whitener (e.g., 10 to 15%), fat reduced cocoa powder (e.g., 15 to 20%), potassium bicarbonate (e.g., 0.1 to 10%), guar gum (e.g., 0.06 to 2%), carrageenan (e.g., 0.05 to 5%), flavors (e.g., chocolate and/or vanilla), and monatin. For example, a dry chocolate drink composition can include 26% skimmed milk powder, 40% whey powder, 12% coffee whitener, 18% fat reduced cocoa powder, 1% potassium bicarbonate, 0.6% guar gum, 0.5% carrageenan, chocolate flavor, vanilla flavor, and monatin. In another embodiment, to prepare a chocolate beverage, the premix can be reconstituted with water or milk such that the resulting beverage contains about 0.5 to 1.5 % (e.g., 0.8%) of the mix.

In some embodiments, mixtures of dry ingredients useful in preparing a beverage composition, mixtures of wet ingredients useful for the same, or liquid mixtures (dispersions) of dry and wet ingredients, are provided as compositions. Such compositions may be provided as an article of manufacture and can be packaged in appropriate containers (e.g., bags, buckets, cartons) for easy transport to points of sale and preparation and for easy pouring and/or mixing. The article of manufacture may contain optional objects, such as utensils; containers for mixing; or other optional ingredients. The articles of manufacture can include instructions for preparing beverage compositions.

It is expected that monatin contained in beverages, as compared to other sweeteners in beverages, will have a longer shelf-life, greater heat and acid stability, as well as better taste characteristics and marketing advantages. The invention will be further described in the following examples, which does not limit the scope of the invention described.

EXAMPLES

EXAMPLE 1

Cloning and Expression of Tryptophan Aminotransferases

This example describes methods that were used to clone tryptophan aminotransferases, which can be used to convert tryptophan to indole-3-pyruvate.

Experimental Overview

Eleven genes encoding aminotransferases were cloned into *E. coli*. These genes were *Bacillus subtilis* D-alanine aminotransferase (*dat*, Genbank Accession No. Y14082.1 bp 28622-29470 and Genbank Accession No. NP_388848.1, nucleic acid sequence and amino acid sequence, respectively), *Sinorhizobium meliloti* (also termed *Rhizobium meliloti*) tyrosine aminotransferase (*tatA*, SEQ ID NOS: 1 and 2, nucleic acid sequence and amino acid sequence, respectively), *Rhodobacter sphaeroides* strain 2.4.1 tyrosine aminotransferase (*tatA* asserted by homology, SEQ ID NOS: 3 and 4, nucleic acid sequence and amino acid sequence, respectively), *R. sphaeroides* 35053 tyrosine aminotransferase (asserted by homology, SEQ ID NOS: 5 and 6, nucleic acid sequence and amino acid sequence, respectively), *Leishmania major* broad substrate aminotransferase (*bsat*, asserted by homology to peptide fragments from *L. mexicana,* SEQ ID NOS: 7 and 8, nucleic acid sequence and amino acid sequence, respectively), *Bacillus subtilis* aromatic aminotransferase (*araT*, asserted by homology, SEQ ID NOS: 9 and 10, nucleic acid sequence and amino acid sequence, respectively), *Lactobacillus amylovorus* aromatic aminotransferase (*araT* asserted by homology, SEQ ID NOS: 11 and 12, nucleic acid sequence and amino acid sequence, respectively), *R. sphaeroides* 35053 multiple substrate aminotransferase (asserted by homology, SEQ ID NOS: 13 and 14, nucleic acid sequence and amino acid sequence, respectively), *Rhodobacter sphaeroides* strain 2.4.1 multiple substrate aminotransferase (*msa* asserted by homology, Genbank Accession No. AAAE01000093.1, bp 14743-16155 and Genbank Accession No. ZP00005082.1, nucleic acid sequence and amino acid sequence, respectively), *Escherichia coli* aspartate aminotransferase (*aspC*, Genbank Accession No. AE000195.1 bp 2755-1565 and Genbank Accession No. AAC74014.1, nucleic acid sequence and amino acid sequence, respectively), and *E. coli* tyrosine aminotransferase (*tyrB*, SEQ ID NOS: 31 and 32, nucleic acid sequence and amino acid sequence, respectively).

The genes were cloned, expressed, and tested for activity in conversion of tryptophan to indole-3-pyruvate, along with commercially available enzymes. All eleven clones had activity.

Identification of Bacterial Strains that Can Contain Polypeptides with the Desired Activity

No genes in the NCBI (National Center for Biotechnology Information) database were designated as tryptophan aminotransferases. However, organisms having this enzymatic activity have been identified. L-tryptophan aminotransferase (TAT) activity has been measured in cell extracts or from purified protein from the following sources: Rhizobacterial isolate from *Festuca octoflora*, pea mitochondria and cytosol, sunflower crown gall cells, *Rhizobium leguminosarum* biovar *trifoli*, *Erwinia herbicola* pv gypsophilae, *Pseudomonas syringae* pv. savastanoi, *Agrobacterium tumefaciens*, *Azospirillum lipferum & brasilense*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Bradyrhizobium elkanii*, *Candida maltosa*, *Azotobacter vinelandii*, rat brain, rat liver, *Sinorhizobium meliloti*, *Pseudomonas fluorescens* CHA0, *Lactococcus lactis*, *Lactobacillus casei, Lactobacillus helveticus*, wheat seedlings, barley, *Phaseolus aureus* (mung bean), *Saccharomyces uvarum* (carlsbergensis), *Leishmania* sp., maize, tomato shoots, pea plants, tobacco, pig, *Clostridium sporogenes*, and *Streptomyces griseus*.

EXAMPLE 2

Conversion of Indole-3-lactate to Indole-3-pyruvate

As shown in FIGS. 1 and 3, indole-3-lactic acid can be used to produce indole-3-pyruvate. Conversion between lactic acid and pyruvate is a reversible reaction, as is conversion between indole-3-pyruvate and indole-3-lactate. The oxidation of indole-lactate was typically followed due to the high amount of background at 340 nm from indole-3-pyruvate.

The standard assay mixture contained 100 mM potassium phosphate, pH 8.0, 0.3 mM NAD^+, 7 units of lactate dehydrogenase (LDH) (Sigma-L2395, St. Louis, MO), and 2 mM substrate in 0.1 mL. The assay was performed in duplicate in a UV-transparent microtiter plate, using a Molecular Devices SpectraMax Plus platereader. Polypeptide and buffer were mixed and pipetted into wells containing the indole-3-lactic acid and NAD^+ and the absorbance at 340 nm of each well was read at intervals of 9 seconds after brief mixing. The reaction was held at 25°C for 5 minutes. The increase in absorbance at 340 nm follows the production of

NADH from NAD^+. Separate negative controls were performed without NAD^+ and without substrate. D-LDH from *Leuconostoc mesenteroides* (Sigma catalog number L2395) appeared to exhibit more activity with the indole-derivative substrates than did L-LDH from *Bacillus stearothermophilus* (Sigma catalog number L5275).

Similar methods were utilized with D-lactic acid and NAD+ or NADH and pyruvate, the natural substrates of D-LDH polypeptides. The V_{max} for the reduction of pyruvate was 100-1000 fold higher than the V_{max} for the oxidation of lactate. The V_{max} for the oxidation reaction of indole-3-lactic with D-LDH was approximately one-fifth of that with lactic acid. The presence of indole-3-pyruvate was also measured by following the change in absorbance at 327 (the enol-borate derivative) using 50 mM sodium borate buffer containing 0.5 mM EDTA and 0.5 mM sodium arsenate. Small, but repeatable, absorbance changes were observed, as compared to the negative controls for both L and D-LDH polypeptides.

Additionally, broad specificity lactate dehydrogenases (enzymes with activity associated with EC 1.1.1.27, EC 1.1.1.28, and/or EC 1.1.2.3) can be cloned and used to make indole-3-pyruvate from indole-3-lactic acid. Sources of broad specificity dehydrogenases include *E. coli, Neisseria gonorrhoeae*, and *Lactobacillus plantarum.*

Alternatively, indole-3-pyruvate can be produced by contacting indole-3-lactate with cellular extracts from *Clostridium sporogenes* which contain an indolelactate dehydrogenase (EC 1.1.1.110); or *Trypanosoma cruzi epimastigotes* cellular extracts which contain *p*-hydroxyphenylactate dehydrogenase (EC 1.1.1.222) known to have activity on indole-3-pyruvate; or *Pseudomonas acidovorans* or *E. coli* cellular extracts, which contain an imidazol-5-yl lactate dehydrogenase (EC 1.1.1.111); or *Coleus blumei*, which contains a hydroxyphenylpyruvate reductase (EC 1.1.1.237); or *Candida maltosa* which contains a D-aromatic lactate dehydrogenase (EC 1.1.1.222). References describing such activities include, Nowicki *et al.* (*FEMS Microbiol Lett* 71:119-24, 1992), Jean and DeMoss (*Canadian J. Microbiol.* 14 1968, Coote and Hassall (*Biochem. J.* 111: 237-9, 1969), Cortese *et al.* (*C.R. Seances Soc. Biol. Fil.* 162 390-5, 1968), Petersen and Alfermann (*Z. Naturforsch. C: Biosci.* 43 501-4, 1988), and Bhatnagar *et al.* (*J. Gen Microbiol* 135:353-60, 1989). In addition, a lactate oxidase such as the one from *Pseudomonas sp.* (Gu *et al.* J. Mol. Catalysis B: Enzymatic: 18:299-305, 2002), can be utilized for oxidation of indole-3-lactic to indole-3-pyruvate.

EXAMPLE 3

Conversion of L-tryptophan to Indole-3-pyruvate utilizing L-amino acid oxidase

This example describes methods used to convert tryptophan to indole-3-pyruvate via an oxidase (EC 1.4.3.2), as an alternative to using a tryptophan aminotransferase as described in Example 1. L-amino acid oxidase was purified from *Crotalus durissus* (Sigma, St. Louis, MO, catalog number A-2805). The accession numbers of L-amino acid oxidases for molecular cloning include: CAD21325.1, AAL14831, NP_490275, BAB78253, A38314, CAB71136, JE0266, T08202, S48644, CAC00499, P56742, P81383, O93364, P81382, P81375, S62692, P23623, AAD45200, AAC32267, CAA88452, AP003600, and Z48565.

Reactions were performed in microcentrifuge tubes in a total volume of 1 mL, incubated for 10 minutes while shaking at 37°C. The reaction mix contained 5 mM L-tryptophan, 100 mM sodium phosphate buffer pH 6.6, 0.5 mM sodium arsenate, 0.5 mM EDTA, 25 mM sodium tetraborate, 0.016 mg catalase (83 U, Sigma C-3515), 0.008 mg FAD (Sigma), and 0.005-0.125 Units of L-amino acid oxidase. Negative controls contained all components except tryptophan, and blanks contained all components except the oxidase. Catalase was used to remove the hydrogen peroxide formed during the oxidative deamination. The sodium tetraborate and arsenate were used to stabilize the enol-borate form of indole-3-pyruvate, which shows a maximum absorbance at 327 nm. Indole-3-pyruvate standards were prepared at concentrations of 0.1-1 mM in the reaction mix.

The purchased L-amino acid oxidase had a specific activity of 540 μg indole-3-pyruvate formed per minute per mg protein. This is the same order of magnitude as the specific activity of tryptophan aminotransferase enzymes.

EXAMPLE 4

Converting Indole-3-pyruvate to 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid with an Aldolase

This example describes methods that can be used to convert indole-3-pyruvate to MP using an aldolase (lyase) (FIG. 2). Aldol condensations are reactions that form carbon-carbon bonds between the β-carbon of an aldehyde or ketone and the carbonyl carbon of another aldehyde or ketone. A carbanion is formed on the carbon adjacent to the carbonyl group of one substrate, and serves as a nucleophile attacking the carbonyl carbon of the second substrate (the electrophilic carbon). Most commonly, the electrophilic substrate is an

aldehyde, so most aldolases fall into the EC 4.1.2.- category. Quite often, the nucleophilic substrate is pyruvate. It is less common for aldolases to catalyze the condensation between two keto-acids or two aldehydes.

However, aldolases that catalyze the condensation of two carboxylic acids have been identified. For example, EP 1045-029 describes the production of L-4-hydroxy-2-ketoglutaric acid from glyoxylic acid and pyruvate using a *Pseudomonas* culture (EC 4.1.3.16). In addition, 4-hydroxy-4-methyl-2-oxoglutarate aldolase (4-hydroxy-4-methyl-2-oxoglutarate pyruvate lyase, EC 4.1.3.17) can catalyze the condensation of two keto acids. Therefore, similar aldolase polypeptides were used to catalyze the condensation of indole-3-pyruvate with pyruvate.

Cloning

4-Hydroxy-4-methyl-2-oxoglutarate pyruvate lyases (ProA aldolase, EC 4.1.3.17) and 4-hydroxy-2-oxoglutarate glyoxylate-lyase (KHG aldolase, EC 4.1.3.16) catalyze reactions very similar to the aldolase reaction of FIG. 2. Primers were designed with compatible overhangs for the pET30 Xa/LIC vector (Novagen, Madison, WI).

Activity Results with proA *gene products*

Both the *C. testosteroni proA* and *S. meliloti SMc00502* gene constructs had high levels of expression when induced with IPTG. The recombinant proteins were highly soluble, as determined by SDS-PAGE analysis of total protein and cellular extract samples. The *C. testosteroni* gene product was purified to > 95% purity. Because the yield of the *S. meliloti* gene product was very low after affinity purification using a His-Bind cartridge, cellular extract was used for the enzymatic assays.

Both recombinant aldolases catalyzed the formation of MP from indole-3-pyruvate and pyruvate. The presence of both divalent magnesium and potassium phosphate were required for enzymatic activity. No product was apparent when indole-3-pyruvate, pyruvate, or potassium phosphate was absent. A small amount of the product was also formed in the absence of enzyme (typically one order of magnitude less than when enzyme was present).

The product peak eluted from the reverse phase C18 column slightly later than the indole-3-pyruvate standard, the mass spectrum of this peak showed a collisionally-induced parent ion ([M + H]+) of 292.1, the parent ion expected for the product MP. The major daughter fragments present in the mass spectrum included those with m/z =158 (1*H*-indole-3-carbaldehyde carbonium ion), 168 (3-buta-1,3-dienyl-1*H*-indole carbonium ion), 274 (292 - H_2O), 256 (292 - 2 H_2O), 238 (292 - 3 H2O), 228 (292 - CH4O3), and 204 (loss of pyruvate). The product also exhibited a UV spectrum characteristic of other indole-containing compounds such as tryptophan, with the λ_{max} of 279-280 and a small shoulder at approximately 290 nm.

The amount of MP produced by the *C. testosteroni* aldolase increased with an increase in reaction temperature from room temperature to 37°C, amount of substrate, and amount of magnesium. The synthetic activity of the enzyme decreased with increasing pH, the maximum product observed was at pH 7. Based on tryptophan standards, the amount of MP produced under a standard assay using 20 µg of purified protein was approximately 10-40 µg per one mL reaction.

Due to the high degree of homology of the *S. meliloti* and *C. testosteroni* ProA aldolase coding sequences with the other genes described above, it is expected that all of the recombinant gene products can catalyze this reaction. Moreover, it is expected that aldolases that have threonine (T) at positions 59 and 87, arginine (R) at 119, aspartate (D) at 120, and histidine (H) at 31 and 71, (based on the numbering system of *C. testosteroni*) will have similar activity.

Activity Results with khg *gene products*

Both the *B. subtilis* and *E. coli khg* gene constructs had high levels of expression of protein when induced with IPTG, while the *S. meliloti khg* had a lower level of expression. The recombinant proteins were highly soluble, as judged by SDS-PAGE analysis of total proteins and cellular extracts. The *B. subtilis* and *E. coli khg* gene products were purified to > 95% purity; the yield of the *S. meliloti* gene product was not as high after affinity purification using a His-Bind cartridge.

There is no evidence that magnesium and phosphate are required for activity for this enzyme. However, the literature reports performing the assays in sodium phosphate buffer, and the enzyme reportedly is bifunctional and has activity on phosphorylated substrates such as 2-keto-3-deoxy-6-phosphogluconate (KDPG). The enzymatic assays were performed as described above, and in some instances the phosphate was omitted. The results indicate that the recombinant KHG aldolases produced MP, but were not as active as the ProA aldolases. In some cases the level of MP produced by KHG was almost identical to the amount produced by magnesium and phosphate alone. Phosphate did not appear to increase the KHG activities. The *Bacillus* enzyme had the highest activity, approximately 20-25% higher activity than the magnesium and phosphate alone, as determined by SRM (see Example 10). The *Sinorhizobium* enzyme had the least amount of activity, which can be associated with folding and solubility problems noted in the expression. All three enzymes have the active site glutamate (position 43 in *B. subtilis* numbering system) as well as the lysine required for Shiff base formation with pyruvate (position 130); however, the *B. subtilis* enzyme contains a threonine in position 47, an active site residue, rather than arginine. The *B. subtilis* KHG is smaller and appears to be in a cluster distinct from the *S. meliloti* and *E. coli* enzymes, with other enzymes having the active site threonine. The differences in the active site may be the reason for the increased activity of the *B. subtilis* enzyme.

Improvement of Aldolase Activity

Catalytic antibodies can be as efficient as natural aldolases, accept a broad range of substrates, and can be used to catalyze the reaction shown in FIG. 2.

Aldolases can also be improved by directed evolution, for example as previously described for a KDPG aldolase (highly homologous to KHG described above) evolved by DNA shuffling and error-prone PCR to remove the requirement for phosphate and to invert the enantioselectivity. The KDPG aldolase polypeptides are useful in biochemical reactions since they are highly specific for the donor substrate (herein, pyruvate), but are relatively flexible with respect to the acceptor substrate (i.e. indole-3-pyruvate) (Koeller & Wong, *Nature* 409:232-9, 2001). KHG aldolase has activity for condensation of pyruvate with a number of carboxylic acids. Mammalian versions of the KHG aldolase are thought to have broader specificity than bacterial versions, including higher activity on 4-hydroxy 4-methyl 2-oxoglutarate and acceptance of both stereoisomers of 4-hydroxy-2-ketoglutarate. Bacterial

sources appear to have a 10-fold preference for the R stereoisomer. There are nearly 100 KHG homologs available in genomic databases, and activity has been demonstrated in *Pseudomonas, Paracoccus, Providencia, Sinorhizobium, Morganella, E. coli,* and mammalian tissues. These enzymes can be used as a starting point for tailoring the enantiospecificity that is desired for monatin production.

Aldolases that utilize pyruvate and another substrate that is either a keto acid and/or has a bulky hydrophobic group like indole can be "evolved" to tailor the polypeptide's specificity, speed, and selectivity. In addition to KHG and ProA aldolases demonstrated herein, examples of these enzymes include, but are not limited to: KDPG aldolase and related polypeptides (KDPH); transcarboxybenzalpyruvate hydratase-aldolase from *Nocardioides* st; 4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase (2'-carboxybenzalpyruvate aldolase) which condenses pyruvate and 2-carboxybenzaldehyde (an aromatic ring-containing substrate); trans-O-hydroxybenzylidenepyruvate hydratase-aldolase from *Pseudomonas putida* and *Sphingomonas aromaticivorans*, which also utilizes pyruvate and an aromatic-containing aldehyde as substrates; 3-hydroxyaspartate aldolase (erythro-3-hydroxy-L-aspartate glyoxylate lyase), which uses 2-oxo acids as the substrates and is thought to be in the organism *Micrococcus denitrificans*; benzoin aldolase (benzaldehyde lyase), which utilizes substrates containing benzyl groups; dihydroneopterin aldolase; L-threo-3-phenylserine benzaldehyde-lyase (phenylserine aldolase) which condenses glycine with benzaldehyde; 4-hydroxy-2-oxovalerate aldolase; 1,2-dihydroxybenzylpyruvate aldolase; and 2-hydroxybenzalpyruvate aldolase.

A polypeptide having the desired activity can be selected by screening clones of interest using the following methods. Tryptophan auxotrophs are transformed with vectors carrying the clones of interest on an expression cassette and are grown on a medium containing small amounts of monatin or MP. Since aminotransferases and aldolase reactions are reversible, the cells are able to produce tryptophan from a racemic mixture of monatin. Similarly, organisms (both recombinant and wildtype) can be screened by ability to utilize MP or monatin as a carbon and energy source. One source of target aldolases is expression libraries of various *Pseudomonas* and rhizobacterial strains. Pseudomonads have many unusual catabolic pathways for degradation of aromatic molecules and they also contain many aldolases; whereas the rhizobacteria contain aldolases, are known to grow in the plant

rhizosphere, and have many of the genes described for construction of a biosynthetic pathway for monatin.

EXAMPLE 5

Chemical Synthesis of the Monatin Precursor

Example 4 described a method of using an aldolase to convert indole-3-pyruvate to MP. This example describes an alternative method of chemically synthesizing MP. MP can be formed using a typical aldol-type condensation (FIG. 4). Briefly, a typical aldol-type reaction involves the generation of a carbanion of the pyruvate ester using a strong base, such as LDA (lithium diisopropylamide), lithium hexamethyldisilazane or butyl lithium. The carbanion that is generated reacts with the indole-pyruvate to form the coupled product.

Protecting groups that can be used for protecting the indole nitrogen include, but are not limited to: t-butyloxycarbonyl (Boc), and benzyloxycarbonyl (Cbz). Blocking groups for carboxylic acids include, but are not limited to, alkyl esters (for example, methyl, ethyl, benzyl esters). When such protecting groups are used, it is not possible to control the stereochemistry of the product that is formed. However, if R2 and/or R3 are chiral protecting groups (FIG. 4), such as (S)-2-butanol, menthol, or a chiral amine, this can favor the formation of one MP enantiomer over the other.

EXAMPLE 6

Conversion of Tryptophan or Indole-3-Pyruvate to Monatin

An *in vitro* process utilizing two enzymes, an aminotransferase and an aldolase, produced monatin from tryptophan and pyruvate. In the first step alpha-ketoglutarate was the acceptor of the amino group from tryptophan in a transamination reaction generating indole-3-pyruvate and glutamate. An aldolase catalyzed the second reaction in which pyruvate was reacted with indole-3-pyruvate, in the presence of Mg^{2+} and phosphate, generating the alpha-keto derivative of monatin (MP), 2-hydroxy-2-(indol-3-ylmethyl)-4-ketoglutaric acid. Transfer of the amino group from the glutamate formed in the first reaction produced the desired product, monatin. Purification and characterization of the product established that the stereoisomer formed was S,S-monatin. Alternative substrates, enzymes, and conditions are described as well as improvements that were made to this process.

Enzymes

The aldolase, 4-hydroxy-4-methyl-2-oxoglutarate pyruvate lyase (ProA aldolase, *proA* gene) (EC 4.1.3.17) from *Comamonas testosteroni* was cloned, expressed and purified as described in Example 4. The 4-hydroxy-2-oxoglutarate glyoxylate lyases (KHG aldolases) (EC 4.1.3.16) from *B. subtilis*, *E. coli*, and *S. meliloti* were cloned, expressed and purified as described in Example 4.

The aminotransferases used in conjunction with the aldolases to produce monatin were L-aspartate aminotransferase encoded by the *E. coli aspC* gene, the tyrosine aminotransferase encoded by the *E. coli tyrB* gene, the *S. meliloti* TatA enzyme, the broad substrate aminotransferase encoded by the *L. major bsat* gene, or the glutamic-oxaloacetic transaminase from pig heart (Type IIa). The cloning, expression and purification of the non-mammalian proteins are described in Example 1. Glutamic-oxaloacetic transaminase from pig heart (type IIa) was obtained from Sigma (# G7005).

Method using ProA aldolase and L-aspartate aminotransferase

The reaction mixture contained 50 mM ammonium acetate, pH 8.0, 4 mM $MgCl_2$, 3 mM potassium phosphate, 0.05 mM pyridoxal phosphate, 100 mM ammonium pyruvate, 50 mM tryptophan, 10 mM alpha-ketoglutarate, 160 mg of recombinant *C. testosteroni* ProA aldolase (unpurified cell extract, ~30% aldolase), 233 mg of recombinant *E. coli* L-aspartate aminotransferase (unpurified cell extract, ~40% aminotransferase) in one liter. All components except the enzymes were mixed together and incubated at 30°C until the tryptophan dissolved. The enzymes were then added and the reaction solution was incubated at 30°C with gentle shaking (100 rpm) for 3.5 hours. At 0.5 and 1 hour after the addition of the enzymes aliquots of solid tryptophan (50 mmoles each) were added to the reaction. All of the added tryptophan did not dissolve, but the concentration was maintained at 50 mM or higher. After 3.5 hours, the solid tryptophan was filtered off. Analysis of the reaction mixture by LC/MS using a defined amount of tryptophan as a standard showed that the concentration of tryptophan in the solution was 60.5 mM and the concentration of monatin was 5.81 mM (1.05 g).

The following methods were used to purify the final product. Ninety percent of the clear solution was applied to a column of BioRad AG50W-X8 resin (225 mL; binding capacity of

1.7 meq/mL). The column was washed with water, collecting 300 mL fractions, until the absorbance at 280 nm was <5% of the first flow through fraction. The column was then eluted with 1 M ammonium acetate, pH 8.4, collecting 4 300-mL fractions. All 4 fractions contained monatin and were evaporated to 105 mL using a roto-evaporator with a tepid water bath. A precipitate formed as the volume reduced and was filtered off over the course of the evaporation process.

Analysis of the column fractions by LC/MS showed that 99% of the tryptophan and monatin bound to the column. The precipitate that formed during the evaporation process contained >97% tryptophan and <2% of monatin. The ratio of tryptophan to product in the supernatant was approximately 2:1.

The supernatant (7 mL) was applied to a 100 mL Fast Flow DEAE Sepharose (Amersham Biosciences) column previously converted to the acetate form by washing with 0.5 L 1 M NaOH, 0.2 L water, 1.0 L of 1.0 M ammonium acetate, pH 8.4, and 0.5 L water. The supernatant was loaded at <2 mL/min and the column was washed with water at 3-4 mL/min until the absorbance at 280 nm was ~0. Monatin was eluted with 100 mM ammonium acetate, pH 8.4, collecting 4 100-mL fractions.

Analysis of the fractions showed that the ratio of tryptophan to monatin in the flow through fractions was 85:15 and the ratio in the eluent fractions was 7:93. Assuming the extinction coefficient at 280 nm of monatin is the same as tryptophan, the eluent fractions contained 0.146 mmole of product. Extrapolation to the total 1 L reaction would produce ~2.4 mmoles (~710 mg) of monatin, for a recovery of 68%.

The eluent fractions from the DEAE Sepharose column were evaporated to <20 mL. An aliquot of the product was further purified by application to a C_8 preparative reversed-phase column using the same chromatographic conditions as those described in Example 10 for the analytical-scale monatin characterization. Waters Fractionlynx™ software was employed to trigger automated fraction collection of monatin based on detection of the $m/z = 293$ ion. The fraction from the C_8 column with the corresponding protonated molecular ion for monatin was collected, evaporated to dryness, and then dissolved in a small volume of water. This fraction was used for characterization of the product.

The resulting product was characterized using the following methods.

UV/Visible Spectroscopy. UV/visible spectroscopic measurements of monatin produced enzymatically were carried out using a Cary 100 Bio UV/visible spectrophotometer. The purified product, dissolved in water, showed an absorption maximum of 280 nm with a shoulder at 288 nm, characteristics typical of indole containing compounds.

LC/MS Analysis. Analyses of mixtures for monatin derived from the *in vitro* biochemical reactions were carried out as described in Example 10. A typical LC/MS analysis of monatin in an *in vitro* enzymatic synthetic mixture is illustrated in FIG. 5. The lower panel of FIG. 5 illustrates a selected ion chromatogram for the protonated molecular ion of monatin at m/z = 293. This identification of monatin in the mixture was corroborated by the mass spectrum illustrated in FIG. 6. Analysis of the purified product by LC/MS showed a single peak with a molecular ion of 293 and absorbance at 280 nm. The mass spectrum was identical to that shown in FIG. 6.

MS/MS Analysis. LC/MS/MS daughter ion experiments, as described in Example 10, were also performed on monatin. A daughter ion mass spectrum of monatin is illustrated in FIG. 7. Tentative structural assignments of all fragment ions labeled in FIG. 7 were made. These include fragment ions of m/z = 275 (293 – H_2O), 257 (293-(2 x H_2O)), 230 (275-COOH), 212 (257-COOH), 168 (3-buta-1,3-dienyl-1*H*-indole carbonium ion), 158 (1*H*-indole-3-carbaldehyde carbonium ion), 144 (3-ethyl-1*H*-indole carbonium ion), 130 (3-methylene-1*H*-indole carbonium ion), and 118 (indole carbonium ion). Many of these are the same as those obtained for MP (Example 4), as expected if derived from the indole portion of the molecule. Some are 1 mass unit higher than those seen for MP, due to the presence of an amino group instead of a ketone.

Accurate Mass Measurement of Monatin. FIG. 8 illustrates the mass spectrum obtained for purified monatin employing an Applied Biosystems-Perkin Elmer Q-Star hybrid quadrupole/time-of-flight mass spectrometer. The measured mass for protonated monatin using tryptophan as an internal mass calibration standard was 293.1144. The calculated mass of protonated monatin, based on the elemental composition $C_{14}H_{17}N_2O_5$ is 293.1137. This is

a mass measurement error of less than 2 parts per million (ppm), providing conclusive evidence of the elemental composition of monatin produced enzymatically.

NMR Spectroscopy. The NMR experiments were performed on a Varian Inova 500 MHz instrument. The sample of monatin (~3 mg) was dissolved in 0.5 mL of D_2O. Initially, the solvent (D_2O) was used as the internal reference at 4.78 ppm. Since the peak for water was large, the ^{1}H-NMR was run with suppression of the peak for water. Subsequently, due to the broadness of the water peak, the C-2 proton of monatin was used as the reference peak, and set at the published value of 7.192 ppm.

For ^{13}C-NMR, an initial run of several hundred scans indicated that the sample was too dilute to obtain an adequate ^{13}C spectrum in the allotted time. Therefore, a heteronuclear multiple quantum coherence (HMQC) experiment was performed, which enabled the correlation of the hydrogens and the carbons to which they were attached, and also providing information on the chemical shifts of the carbons.

A summary of the ^{1}H and HMQC data is shown in Tables 1 and 2. By comparison to published values, the NMR data indicated that the enzymatically produced monatin was either (S,S), (R,R), or a mixture of both.

Chiral LC/MS Analysis. To establish that the monatin produced *in vitro* was one stereoisomer, and not a mixture of the (R,R) and (S,S) enantiomers, chiral LC/MS analyses were carried out using the instrumentation described in Example 10.

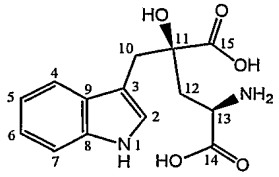
Chiral LC separations were made using an Chirobiotic T (Advanced Separations Technology) chiral chromatography column at room temperature. Separation and detection, based on published protocols from the vendor, were optimized for the R- (D) and S- (L) stereoisomers of tryptophan. The LC mobile phase consisted of A) water containing 0.05% (v/v) trifluoroacetic acid; B) Methanol containing 0.05% (v/v) trifluoroacetic acid. The elution was isocratic at 70% A and 30% B. The flow rate was 1.0 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. The instrumental parameters used for chiral LC/MS analysis of tryptophan and monatin are identical to those described in Example 10 for LC/MS analysis. Collection of mass spectra for the region *m/z* 150–400 was utilized. Selected ion

chromatograms for protonated molecular ions ($[M + H]^+ = 205$ for both R- and S-tryptophan and $[M + H]^+ = 293$ for monatin) allowed direct identification of these analytes in the mixtures.

The chromatograms of R- and S-tryptophan and monatin, separated by chiral chromatography and monitored by MS, are shown in FIG. 9. The single peak in the chromatogram of monatin indicates that the compound is one stereoisomer, with a retention time almost identical to S-tryptophan.

TABLE 1

^{1}H NMR data



	Cargill		Vleggaar et al.[1]		Takeshi et al.[2]	
Atom	**δ_H**	**J(HH) Hz**	**δ_H**	**J(HH) Hz**	**δ_H**	**J(HH) Hz**
2	7.192 (1H, s)		7.192 (s)		7.18 (s)	
4	7.671 (d)	7.99	7.686 (d)	7.9	7.67 (d)	8.0
5	7.104 (dd)	7.99	7.102 (dd)	8.0, 8.0	7.11 (dd)	7.5, 7.5
6	7.178 (dd)	*	7.176 (dd)	8.0, 8.0	7.17 (dd)	7.5, 7.5
7	7.439 (d)	7.99	7.439 (d)	8.1	7.43 (d)	8.0
10a	3.242(d)	14.5	3.243 (d)	14.3	3.24 (d)	14.5
10b	3.033 (d)	14.5	3.051 (d)	14.3	3.05 (d)	14.5
12	2.626 (dd) 2.015 (dd)	15.5, 1.5 15.0, 12.0	2.651 (dd) 2.006 (dd)	15.3, 1.7 15.3, 11.7	2.62 (dd) 2.01 (dd)	15.5, 1.8 15.5, 12.0
13	3.571 (dd)	10.75*, 1.5	3.168 (dd)	11.6, 1.8	3.57 (dd)	12.0, 1.8

[1] Vleggaar *et al.* (*J.C.S. Perkin Trans.* 1:3095-8, 1992).
[2] Takeshi and Shusuke (JP2002060382, 2002-02-26).

TABLE 2

^{13}C NMR data (from HMQC spectrum)

	Cargill	**Vleggaar et al.[1]**
Atom	**δ_C**	**δ_C**
2	126.1	126.03
3	*	110.31
4	120.4	120.46
5	120.2	120.25
6	122.8	122.74
7	112.8	112.79
8	*	137.06
9	*	129.23
10a	36.4	36.53
12	39.5	39.31
13	54.9	54.89
14	*	175.30
15	*	181.18

[1] Vleggaar *et al.* (*J.C.S. Perkin Trans.* 1:3095-8, 1992).

Polarimetry. The optical rotation was measured on a Rudolph Autopol III polarimeter. The monatin was prepared as a 14.6 mg/mL solution in water. The expected specific rotation ($[\alpha]_D^{20}$) for S,S monatin (salt form) is -49.6 for a 1 g/mL solution in water (Vleggaar *et al*). The observed $[\alpha]_D^{20}$ was -28.1 for the purified, enzymatically produced monatin indicating that it was the S, S stereoisomer.

Improvements

The reaction conditions, including reagent and enzyme concentrations, were optimized and yields of 5-10 mg/mL were produced using the following reagent mix: 50 mM ammonium acetate pH 8.3, 2 mM $MgCl_2$, 200 mM pyruvate (sodium or ammonium salt), 5 mM alpha-ketoglutarate (sodium salt), 0.05 mM pyridoxal phosphate, deaerated water to achieve a final volume of 1 mL after the addition of the enzymes, 3 mM potassium phosphate, 50 μg/mL of recombinant ProA aldolase (cell extract; total protein concentration of 167 μg/mL), 1000 μg/mL of L-aspartate aminotransferase encoded by the *E. coli aspC* gene (cell extract; total protein concentration of 2500 μg/mL), and solid tryptophan to afford a concentration of > 60 mM (saturated; some undissolved throughout the reaction). The mixture was incubated at 30°C for 4 hours with gentle stirring or mixing.

Substitutions

The concentration of alpha-ketoglutarate can be reduced to 1 mM and supplemented with 9 mM aspartate with an equivalent yield of monatin. Alternative amino acid acceptors can be utilized in the first step, such as oxaloacetate.

When recombinant *L. major* broad substrate aminotransferase was used in place of the *E. coli* L-aspartate aminotransferase, similar yields of monatin were achieved. However, a second unidentified product (3-10% of the major product) with a molecular mass of 292 was also detected by LC-MS analysis. Monatin concentrations of 0.1-0.5 mg/mL were produced when the *E. coli tyrB* encoded enzyme, the *S. meliloti tat A* encoded enzyme or the glutamic-oxaloacetic transaminase from pig heart (type IIa) was added as the aminotransferase. When starting the reaction from indole-3-pyruvate, a reductive amination can be done for the last step with glutamate dehydrogenase and NADH (as in Example 7).

The KHG aldolases from *B. subtilis, E. coli,* and *S. meliloti* were also used with the *E. coli* L-aspartate aminotransferase to produce monatin enzymatically. The following reaction conditions were used: 50 mM NH_4-OAc pH 8.3, 2 mM $MgCl_2$, 200 mM pyruvate, 5 mM glutamate, 0.05 mM pyridoxal phosphate, deaerated water to achieve a final volume of 0.5 mL after the addition of the enzymes, 3 mM potassium phosphate, 20 μg/mL of recombinant *B. subtilis* KHG aldolase (purified), ca. 400 μg/mL of *E. coli* L-aspartate aminotransferase (AspC) unpurified from cell extract, and 12 mM indole-3-pyruvate. The reactions were incubated at 30°C for 30 minutes with shaking. The amount of monatin produced using the *B. subtilis* enzyme was 80 ng/mL, and increased with increasing amounts of aldolase. If indole-3-pyruvate and glutamate were replaced by saturating amounts of tryptophan and 5 mM alpha-ketoglutarate, the production of monatin was increased to 360 ng/mL. Reactions were repeated with 30 μg/mL of each of the three KHG enzymes in 50 mM Tris pH 8.3, with saturating amounts of tryptophan, and were allowed to proceed for an hour in order to increase detection. The *Bacillus* enzyme had the highest activity as in Example 4, producing approximately 4000 ng/mL monatin. The *E. coli* KHG produced 3000 ng/mL monatin, and the *S. meliloti* enzyme produced 2300 ng/mL.

EXAMPLE 7

Interconversion between MP and Monatin

The amination of MP to form monatin can be catalyzed by aminotransferases such as those identified in Examples 1 and 6, or by dehydrogenases that require a reducing cofactor such as NADH or NADPH. These reactions are reversible and can be measured in either direction. The directionality, when using a dehydrogenase enzyme, can be largely controlled by the concentration of ammonium salts.

Dehydrogenase activity. The oxidative deamination of monatin was monitored by following the increase in absorbance at 340 nm as NAD(P)+ was converted to the more chromophoric NAD(P)H. Monatin was enzymatically produced and purified as described in Example 6.

A typical assay mixture contained 50 mM Tris-HCl, pH 8.0 to 8.9, 0.33 mM NAD^+ or $NADP^+$, 2 to 22 units of glutamate dehydrogenase (Sigma), and 10-15 mM substrate in 0.2 mL. The assay was performed in duplicate in a UV-transparent microtiter plate, on a Molecular Devices SpectraMax Plus platereader. A mix of the enzyme, buffer, and $NAD(P)^+$

were pipetted into wells containing the substrate and the increase in absorbance at 340 nm was monitored at 10 second intervals after brief mixing. The reaction was incubated at 25°C for 10 minutes. Negative controls were carried out without the addition of substrate, and glutamate was utilized as a positive control. The type III glutamate dehydrogenase from bovine liver (Sigma # G-7882) catalyzed the conversion of the monatin to the monatin precursor at a rate of conversion approximately one-hundredth the rate of the conversion of glutamate to alpha-ketoglutarate.

Transamination activity. Monatin aminotransferase assays were conducted with the aspartate aminotransferase (AspC) from *E. coli*, the tyrosine aminotransferase (TyrB) from *E. coli*, the broad substrate aminotransferase (BSAT) from *L. major*, and the two commercially available porcine glutamate-oxaloacetate aminotransferases described in Example 1. Both oxaloacetate and alpha-ketoglutarate were tested as the amino acceptor. The assay mixture contained (in 0.5 mL) 50 mM Tris-HCl, pH 8.0, 0.05 mM PLP, 5 mM amino acceptor, 5 mM monatin, and 25 μg of aminotransferase. The assays were incubated at 30°C for 30 minutes, and the reactions were stopped by addition of 0.5 mL isopropyl alcohol. The loss of monatin was monitored by LC/MS (Example 10). The highest amount of activity was noted with *L. major* BSAT with oxaloacetate as the amino acceptor, followed by the same enzyme with alpha-ketoglutarate as the amino acceptor. The relative activity with oxaloacetate was: BSAT > AspC > porcine type IIa > porcine type I =TyrB. The relative activity with alpha-ketoglutarate was: BSAT > AspC > porcine type I > porcine type IIa > TyrB.

EXAMPLE 8

Production of Monatin from Tryptophan and C3 Sources Other than Pyruvate

As described above in Example 6, indole-3-pyruvate or tryptophan can be converted to monatin using pyruvate as the C3 molecule. However, in some circumstances, pyruvate may not be a desirable raw material. For example, pyruvate may be more expensive than other C3 carbon sources, or may have adverse effects on fermentations if added to the medium. Alanine can be transaminated by many PLP-enzymes to produce pyruvate.

Tryptophanase-like enzymes perform beta-elimination reactions at faster rates than other PLP enzymes such as aminotransferases. Enzymes from this class (4.1.99.-) can produce ammonia and pyruvate from amino acids such as L-serine, L-cysteine, and derivatives of

serine and cysteine with good leaving groups such as O-methyl-L-serine, O-benzyl-L-serine, S-methylcysteine, S-benzylcysteine, S-alkyl-L-cysteine, O-acyl-L-serine, 3-chloro-L-alanine.

Processes to produce monatin using EC 4.1.99.- polypeptides can be improved by mutating the β-tyrosinase (TPL) or tryptophanase according to the method of Mouratou *et al.* (*J. Biol. Chem* 274:1320-5, 1999). Mouratou *et al.* describe the ability to covert the β-tyrosinase into a dicarboxylic amino acid β-lyase, which has not been reported to occur in nature. The change in specificity was accomplished by converting valine (V) 283 to arginine (R) and arginine (R) 100 to threonine (T). These amino acid changes allow for the lyase to accept a dicarboxylic amino acid for the hydrolytic deamination reaction (such as aspartate). Aspartate, therefore, can also be used as a source of pyruvate for subsequent aldol condensation reactions.

Additionally, cells or enzymatic reactors can be supplied with lactate and an enzyme that converts lactate to pyruvate. Examples of enzymes capable of catalyzing this reaction include lactate dehydrogenase and lactate oxidase.

The reaction mixture consisted of 50 mM Tris-Cl pH 8.3, 2 mM $MgCl_2$, 200 mM C3 carbon source, 5 mM alpha-ketoglutarate, sodium salt, 0.05 mM pyridoxal phosphate, deaerated water to achieve a final volume of 0.5 mL after the addition of the enzymes, 3 mM potassium phosphate pH 7.5, 25 μg of crude recombinant *C. testosteroni* ProA aldolase as prepared as in Example 4, 500 μg of crude L-aspartate aminotransferase (AspC) as prepared in Example 1, and solid tryptophan to afford a concentration of > 60 mM (saturated; some undissolved throughout the reaction). The reaction mix was incubated at 30°C for 30 minutes with mixing. Serine, alanine, and aspartate were supplied as 3-carbon sources. Assays were performed with and without secondary PLP enzymes (purified) capable of performing beta-elimination and beta-lyase reactions (tryptophanase (TNA), double mutant tryptophanase, β-tyrosinase (TPL)). The results are shown in Table 3:

TABLE 3

Production of monatin utilizing alternative C3-carbon sources

C3-carbon source	Additional PLP Enzyme	Relative Activity
none	None	0%
pyruvate	None	100%
serine	None	3%
serine	11 μg wildtype TNA (1 U)	5.1%
serine	80 μg double mutant TNA	4.6%
alanine	None	32%
alanine	11 μg wildtype TNA	41.7%
alanine	80 μg mutant TNA	43.9%
aspartate	110 μg wildtype TNA (10 U)	7.7%
aspartate	5 U wildtype TPL (crude)	5.1%
aspartate	80 μg mutant TNA	3.3%

The monatin produced from alanine and serine as 3-carbon sources was verified by LC/MS/MS daughter scan analysis, and was identical to the characterized monatin produced in Example 6. Alanine was the best alternative tested, and was transaminated by the AspC enzyme. The amount of monatin produced was increased by addition of the tryptophanase, which is capable of transamination as a secondary activity. The amount of monatin produced with serine as a carbon source nearly doubled with the addition of the tryptophanase enzymes, even though only one-fifth of the amount of tryptophanase was added in comparison to the aminotransferase. AspC is capable of some amount of beta-elimination activity alone. The results with aspartate indicate that the tryptophanase activity on aspartate does not increase with the same site-directed mutations as previously suggested for β-tyrosinase. It is expected that the mutant β-tyrosinase will have higher activity for production of monatin.

EXAMPLE 9

Chemical Synthesis of Monatin

The addition of alanine to indole-3-pyruvic acid produces monatin, and this reaction can be performed synthetically with a Grignard or organolithium reagent.

For example, to 3-chloro- or 3-bromo-alanine which has been appropriately blocked at the carboxyl and amino groups, is added magnesium under anhydrous conditions. Indole-3-pyruvate (appropriately blocked) is then added to form the coupled product followed by

removal of the protecting groups to form monatin. Protecting groups that are particularly useful include THP (tetrahydropyranyl ether) which is easily attached and removed.

EXAMPLE 10

Detection of Tryptophan, Monatin, and MP

This example describes methods used to detect the presence of monatin, or its precursor 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid.

LC/MS Analysis

Analyses of mixtures for monatin, MP, and/or tryptophan derived from *in vitro* or *in vivo* biochemical reactions were performed using a Waters/Micromass liquid chromatography-tandem mass spectrometry (LC/MS/MS) instrument including a Waters 2690 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and a Micromass Quattro Ultima triple quadrupole mass spectrometer. LC separations were made using a Supelco Discovery C_{18} reversed-phase chromatography column, 2.1mm x 150 mm, or an Xterra MS C_8 reversed-phase chromatography column, 2.1mm x 250 mm, at room temperature. The LC mobile phase consisted of A) water containing 0.05% (v/v) trifluoroacetic acid and B) methanol containing 0.05% (v/v) trifluoroacetic acid.

The gradient elution was linear from 5% B to 35% B, 0-9 min, linear from 35% B to 90% B, 9-16 min, isocratic at 90% B, 16-20 min, linear from 90% B to 5% B, 20-22 min, with a 10 min re-equilibration period between runs. The flow rate was 0.25 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of protonated molecular ions ($[M + H]^+$) of the analytes of interest, and production of characteristic fragment ions.

The following instrumental parameters were used for LC/MS analysis of monatin: Capillary: 3.5 kV; Cone: 40 V; Hex 1: 20 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100°C; Desolvation temperature: 350°C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 15.0; High mass resolution (Q1): 15.0; Ion energy: 0.2; Entrance: 50V; Collision Energy: 2; Exit: 50V; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 3.5; Multiplier: 650. Uncertainties for reported mass/charge ratios (*m/z*)

and molecular masses are ± 0.01%. Initial detection of the alpha-keto acid form of monatin (MP) and monatin in the mixtures was accomplished by LC/MS monitoring with collection of mass spectra for the region *m/z* 150–400. Selected ion chromatograms for protonated molecular ions ($[M + H]^+ = 292$ for MP, $[M + H]^+ = 293$ for monatin) allowed direct identification of these analytes in the mixtures.

MS/MS Analysis

LC/MS/MS daughter ion experiments were performed on monatin as follows. A daughter ion analysis involves transmission of the parent ion (e.g., $m/z = 293$ for monatin) of interest from the first mass analyzer (Q1) into the collision cell of the mass spectrometer, where argon is introduced and chemically dissociates the parent into fragment (daughter) ions. These fragment ions are then detected with the second mass analyzer (Q2), and can be used to corroborate the structural assignment of the parent. Tryptophan was characterized and quantified in the same way via transfmission and fragmentation of $m/z = 205$.

The following instrumental parameters were used for LC/MS/MS analysis of monatin: Capillary: 3.5 kV; Cone: 40 V; Hex 1: 20 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 13.0; High mass resolution (Q1): 13.0; Ion energy: 0.2; Entrance: -5 V; Collision Energy: 14; Exit: 1V; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 3.5; Multiplier: 650.

High-Throughput Determination of Monatin and Tryptophan

High-throughput analyses (< 5 min/sample) of mixtures for monatin and tryptophan derived from *in vitro* or *in vivo* reactions were carried out using instrumentation described above, and the same parameters as described for LC/MS/MS. LC separations were made using a 4.6 mm x 50 mm Advanced Separation Technologies Chirobiotic T column at room temperature. The LC mobile phase consisted of A) water containing 0.25% acetic acid; B) Methanol containing 0.25% acetic acid. The isocratic elution was at 50% B, 0-5 min. The flow rate was 0.6 mL/min. All parameters of the ESI-MS/MS system were optimized and selected based on optimal in-source generation of the protonated molecular ion of tryptophan and the internal standard $^2H_5$-tryptophan, as well as collision-induced production of amino acid-specific fragment ions for multiple reaction monitoring (MRM) experiments. The following

instrumental parameters were used for LC/MS/MS analysis of monatin and tryptophan in the positive ion multiple reaction monitoring (mrm) mode: Capillary: 3.5 kV; Cone: 20 V; Hex 1: 15 V; Aperture: 1 V; Hex 2: 0 V; Source temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas: 500 L/h; Cone gas: 40 L/h; Low mass resolution (Q1): 12.0; High mass resolution (Q1): 12.0; Ion energy: 0.2; Entrance: - 5 V; Collision Energy: 14; Exit: 1 V; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 0.5; Multiplier: 650. MRM parameters: Interchannel delay: 0.03 s; Interscan delay: 0.03 s; Dwell: 0.05 s.

Accurate Mass Measurement of Monatin.

High resolution MS analysis was carried out using an Applied Biosystems-Perkin Elmer Q-Star hybrid quadrupole/time-of-flight mass spectrometer. The measured mass for protonated monatin used tryptophan as an internal mass calibration standard. The calculated mass of protonated monatin, based on the elemental composition $C_{14}H_{17}N_2O_5$ is 293.1137. Monatin produced using the biocatalytic process described in Example A showed a measured mass of 293.1144. This is a mass measurement error of less than 2 parts per million (ppm), providing conclusive evidence of the elemental composition of monatin produced enzymatically.

EXAMPLE 11

Production of Monatin in Bacteria

This example describes methods used to produce monatin in *E. coli* cells. One skilled in the art will understand that similar methods can be used to produce monatin in other bacterial cells. In addition, vectors containing other genes in the monatin synthesis pathway (FIG. 2) can be used.

Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol.* 35:200-4, 1990), was prepared as follows. To 700 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 0.2 g $MgSO_4*7H_20$, 0.01 g $CaCl_2*2H_20$, and 0.5 mg $FeSO_4*7H_20$. The pH was adjusted to 7.0, the volume was increased to 850 mL, and the medium was autoclaved. A 50% glucose solution was prepared separately, and sterile-filtered. Forty mL was added to the base medium (850 mL) for a 1 L final volume.

A 10 g/L L-tryptophan solution was prepared in 0.1 M sodium phosphate pH 7, and sterile-filtered. One-tenth volume was typically added to cultures as specified below. A 10% sodium pyruvate solution was also prepared and sterile-filtered. A 10 mL aliquot was typically used per liter of culture. Stocks of ampicillin (100 mg/mL), kanamycin (25 mg/mL) and IPTG (840 mM) were prepared, sterile-filtered, and stored at –20°C before use. Tween 20 (polyoxyethylene 20-Sorbitan monolaurate) was utilized at a 0.2% (vol/vol) final concentration. Ampicillin was used at non-lethal concentrations, typically 1-10 μg/mL final concentration.

Fresh plates of *E. coli* BL21(DE3)::*C. testosteroni proA*/pET 30 Xa/LIC (described in Example 4) were prepared on LB medium containing 50 μg/mL kanamycin. Overnight cultures (5 mL) were inoculated from a single colony and grown at 30°C in LB medium with kanamycin. Typically a 1 to 50 inoculum was used for induction in trp-1 + glucose medium. Fresh antibiotic was added to a final concentration of 50 mg/L. Shake flasks were grown at 37°C prior to induction.

Cells were sampled every hour until an OD_{600} of 0.35-0.8 was obtained. Cells were then induced with 0.1 mM IPTG, and the temperature reduced to 34 °C. Samples (1 mL) were collected prior to induction (zero time point) and centrifuged at 5000 x g. The supernatant was frozen at –20°C for LC/MS analysis. Four hours post-induction, another 1 mL sample was collected, and centrifuged to separate the broth from the cell pellet. Tryptophan, sodium pyruvate, ampicillin, and Tween were added as described above.

The cells were grown for 48 hours post-induction, and another 1 mL sample was taken and prepared as above. At 48 hours, another aliquot of tryptophan and pyruvate were added. The entire culture volume was centrifuged after approximately 70 hours of growth (post-induction), for 20 minutes at 4°C and 3500 rpm. The supernatant was decanted and both the broth and the cells were frozen at –80°C. The broth fractions were filtered and analyzed by LC/MS. The heights and areas of the $[M+H]^+ = 293$ peaks were monitored as described in Example 10. The background level of the medium was subtracted. The data was also normalized for cell growth by plotting the height of the $[M+H]^+ = 293$ peak divided by the optical density of the culture at 600 nm.

Higher levels of monatin were produced when pyruvate, ampicillin, and Tween were added 4 hours post induction rather than at induction. Other additives such as PLP, additional phosphate, or additional $MgCl_2$ did not increase the production of monatin. Higher titers of monatin were obtained when tryptophan was utilized instead of indole-3-pyruvate, and when the tryptophan was added post-induction rather than at inoculation, or at induction. Prior to induction, and 4 hours post-induction (at time of substrate addition), there was typically no detectable level of monatin in the fermentation broth or cellular extracts. Negative controls were done utilizing cells with pET30a vector only, as well as cultures where tryptophan and pyruvate were not added. A parent MS scan demonstrated that the compound with (m+1)/z = 293 was not derived from larger molecules, and daughter scans (performed as in Example 10) were similar to monatin made *in vitro*.

The effect of Tween was studied by utilizing 0, 0.2% (vol/vol), and 0.6% final concentrations of Tween-20. The highest amount of monatin produced by shake flasks was at 0.2% Tween. The ampicillin concentration was varied between 0 and 10 μg/mL. The amount of monatin in the cellular broth increased rapidly (2.5 X) between 0 and 1 μg/mL, and increased 1.3 X when the ampicillin concentration was increased from 1 to 10 μg/mL.

A time course experiment showing typical results is shown in FIG. 10. The amount of monatin secreted into the cell broth increased, even when the values are normalized for cell growth. By using the molar extinction coefficient of tryptophan, the amount of monatin in the broth was estimated to be less than 10 μg/mL. The same experiment was repeated with the cells containing vector without *proA* insert. Many of the numbers were negative, indicating the peak height at m/z=293 was less in these cultures than in the medium alone (FIG. 10). The numbers were consistently lower when tryptophan and pyruvate were absent, demonstrating that monatin production is a result of an enzymatic reaction catalyzed by the aldolase enzyme.

The *in vivo* production of monatin in bacterial cells was repeated in 800 mL shake flask experiments and in fermentors. A 250 mL sample of monatin (in cell-free broth) was purified by anion exchange chromatography and preparative reverse-phase liquid chromatography. This sample was evaporated, and submitted for high resolution mass analysis (described in

Example 6). The high resolution MS indicated that the metabolite being produced is monatin.

In vitro assays indicate that aminotransferase needs to be present at higher levels than aldolase (see Example 6), therefore the aspartate aminotransferase from *E. coli* was overexpressed in combination with the aldolase gene to increase the amount of monatin produced. Primers were designed to introduce *C. testosteroni proA* into an operon with *aspC*/pET30 Xa/LIC, as follows: 5' primer: ACTCGGATCCGAAGGAGATATACATATGTACGAACTGGGACT (SEQ ID NO: 67) and 3' primer: CGGCTGTCGACCGTTAGTCAATATATTTCAGGC (SEQ ID NO: 68). The 5' primer contains a BamHI site, the 3' primer contains a SalI site for cloning. PCR was performed as described in Example 4, and gel purified. The *aspC*/pET30 Xa/LIC construct was digested with BamHI and SalI, as was the PCR product. The digests were purified using a Qiagen spin column. The *proA* PCR product was ligated to the vector using the Roche Rapid DNA Ligation kit (Indianapolis, IN) according to manufacturer's instructions. Chemical transformations were done using Novablues Singles (Novagen) as described in Example 1. Colonies were grown up in LB medium containing 50 mg/L kanamycin and plasmid DNA was purified using the Qiagen spin miniprep kit. Clones were screened by restriction digest analysis and sequence was confirmed by Seqwright (Houston, TX). Constructs were subcloned into BLR(DE3), BLR(DE3)pLysS, BL21(DE3) and BL21(DE3)pLysS (Novagen). The *proA*/pET30 Xa/LIC construct was also transformed into BL21(DE3)pLysS.

Initial comparisons of BLR(DE3) shake flask samples under the standard conditions described above demonstrated that the addition of the second gene (*aspC*) improved the amount of monatin produced by seven-fold. To hasten growth, BL21(DE3)-derived host strains were used. The *proA* clones and the two gene operon clones were induced in Trp-1 medium as above, the pLysS hosts had chloramphenicol (34 mg/L) added to the medium as well. Shake flask experiments were performed with and without the addition of 0.2% Tween-20 and 1 mg/L ampicillin. The amount of monatin in the broth was calculated using *in vitro* produced purified monatin as a standard. SRM analyses were performed as described in Example 10. Cells were sampled at zero, 4 hours, 24 hours, 48 hours, 72 hours, and 96 hours of growth.

The results are shown in Table 4 for the maximum amounts produced in the culture broths. In most instances, the two gene construct gave higher values than the *proA* construct alone. The pLysS strains, which should have leakier cell envelopes, had higher levels of monatin secreted, even though these strains typically grow at a slower rate. The additions of Tween and ampicillin were beneficial.

Table 4

Amount of Monatin Produced by *E. coli* Bacteria

Construct	Host	Tween + Amp	μg/mL monatin	time
proA	BL21(DE3)	-	0.41	72 hr
proA	BL21(DE3)	+	1.58	48 hr
proA	BL21(DE3)pLysS	-	1.04	48 hr
proA	BL21(DE3)pLysS	+	1.60	48 hr
aspC:proA	BL21(DE3)	-	0.09	48 hr
aspC:proA	BL21(DE3)	+	0.58	48 hr
aspC:proA	BL21(DE3)pLysS	-	1.39	48 hr
aspC:proA	BL21(DE3)pLysS	+	6.68	48 hr

EXAMPLE 12

Production of Monatin in Yeast

This example describes methods used to produce monatin in eukaryotic cells. One skilled in the art will understand that similar methods can be used to produce monatin in any cell of interest. In addition, other genes can be used (e.g., those listed in FIG. 2) in addition to, or alternatively to those described in this example.

The pESC Yeast Epitope Tagging Vector System (Stratagene, La Jolla, CA) was used to clone and express the *E. coli aspC* and *C. testosteroni proA* genes into *Saccharomyces cerevisiae*. The pESC vectors contain both the GAL1 and the GAL10 promoters on opposite strands, with two distinct multiple cloning sites, allowing for expression of two genes at the same time. The pESC-His vector also contains the *His3* gene for complementation of histidine auxotrophy in the host (YPH500). The GAL1 and GAL10 promoters are repressed by glucose and induced by galactose; a Kozak sequence is utilized for optimal expression in yeast. The pESC plasmids are shuttle vectors, allowing the initial construct to be made in *E.*

coli (with the *bla* gene for selection); however, no bacterial ribosome binding sites are present in the multiple cloning sites.

The following primers were designed for cloning into pESC-His (restriction sites are underlined, Kozak sequence is in bold): *aspC* (*Bam*HI/*Sal*I), GAL1: 5'-CGCGGATCC**ATAATGG**TTGAGAACATTACCG-3' (SEQ ID NO: 69) and 5'-ACGCGTCGACTTACAGCACTGCCACAATCG-3' (SEQ ID NO: 70). *proA* (*Eco*RI/*Not*I), GAL10: 5'-CCGGAATTC**ATAATGG**TCGAACTGGGAGTTGT-3' (SEQ ID NO: 71) and 5'-GAATGCGGCCGCTTAGTCAATATATTTCAGGCC-3' (SEQ ID NO: 72).

The second codon for both mature proteins was changed from an aromatic amino acid to valine due to the introduction of the Kozak sequence. The genes of interest were amplified using pET30 Xa/LIC miniprep DNA from the clones described in Examples 1 and Example 4 as template. PCR was performed using an Eppendorf Master cycler gradient thermocycler and the following protocol for a 50 µL reaction: 1.0 µL template, 1.0 µM of each primer, 0.4 mM each dNTP, 3.5 U Expand High Fidelity Polymerase (Roche, Indianapolis, IN), and 1X Expand™ buffer with Mg. The thermocycler program used consisted of a hot start at 94°C for 5 minutes, followed by 29 repetitions of the following steps: 94°C for 30 seconds, 50°C for 1 minute 45 seconds, and 72°C for 2 minutes 15 seconds. After the 29 repetitions the sample was maintained at 72°C for 10 minutes and then stored at 4°C. The PCR products were purified by separation on a 1% TAE-agarose gel followed by recovery using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

The pESC-His vector DNA (2.7 µg) was digested with *Bam*HI/*Sal*I and gel-purified as above. The *aspC* PCR product was digested with *Bam*HI/*Sal*I and purified with a QIAquick PCR Purification Column. Ligations were performed with the Roche Rapid DNA Ligation Kit following the manufacturer's protocols. Desalted ligations were electroporated into 40 µl Electromax DH10B competent cells (Invitrogen) in a 0.2 cm Biorad disposable cuvette using a Biorad Gene Pulser II with pulse controller plus, according to the manufacturer's instructions. After 1 hour of recovery in 1 mL of SOC medium, the transformants were plated on LB medium containing 100 µg/mL ampicillin. Plasmid DNA preparations for clones were done using QIAprep Spin Miniprep Kits. Plasmid DNA was screened by

restriction digest, and sequenced (Seqwright) for verification using primers designed for the vector.

The *aspC* /pESC-His clone was digested with *Eco*RI and *Not*I, as was the *proA* PCR product. DNA was purified as above, and ligated as above. The two gene construct was transformed into DH10B cells and screened by restriction digest and DNA sequencing.

The construct was transformed into *S. cerevisiae* strain YPH500 using the S.c. EasyComp™ Transformation Kit (Invitrogen). Transformation reactions were plated on SC-His minimal medium (Invitrogen pYES2 manual) containing 2% glucose. Individual yeast colonies were screened for the presence of the *proA* and *aspC* genes by colony PCR using the PCR primers above. Pelleted cells (2 µl) were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 1 µl of zymolase and heated at 37°C for 10 minutes. Four µL of this suspension was then used in a 50 µL PCR reaction using the PCR reaction mixture and program described above.

Five mL cultures were grown overnight on SC-His + glucose at 30°C and 225 rpm. The cells were gradually adjusted to growth on raffinose in order to minimize the lag period prior to induction with galactose. After approximately 12 hours of growth, absorbance measurements at 600 nm were taken, and an appropriate volume of cells was spun down and resuspended to give an OD of 0.4 in the fresh SC-His medium. The following carbon sources were used sequentially: 1% raffinose + 1 % glucose, 0.5% glucose + 1.5% raffinose, 2% raffinose, and finally 1% raffinose + 2% galactose for induction.

After approximately 16 hours of growth in induction medium, the 50 mL cultures were divided into duplicate 25 mL cultures, and the following were added to only one of the duplicates: (final concentrations) 1 g/L L-tryptophan, 5 mM sodium phosphate pH 7.1, 1 g/L sodium pyruvate, 1 mM $MgCl_2$. Samples of broths and cell pellets from the non-induction medium, and from the 16 hour cultures prior to addition of substrates for the monatin pathway, were saved as negative controls. In addition, constructs containing only a functional *aspC* gene (and a truncated *proA* gene) were utilized as another negative control. The cells were allowed to grow for a total of 69 hours post-induction. Occasionally the yeast cells were induced at a lower OD, and only grown for 4 hours prior to addition of tryptophan

and pyruvate. However, these monatin substrates appear to inhibit growth and the addition at higher OD was more effective.

The cell pellets from the cultures were lysed with 5 mL of YeastBuster™ + 50 µl THP (Novagen) per gram (wet weight) of cells following manufacturer's protocols, with the addition of protease inhibitors and benzonase nuclease as described in previous examples. The culture broth and cell extracts were filtered and analyzed by SRM as described in Example 10. Using this method, no monatin was detected in the broth samples, indicating that the cells could not secrete monatin under these conditions. The proton motive force may be insufficient under these conditions or the general amino acid transporters may be saturated with tryptophan. Protein expression was not at a level that allowed for detection of changes using SDS-PAGE.

Monatin was detectable (approximately 60 ng/mL) transiently in cell extracts of the culture with two functional genes, when tryptophan and pyruvate were added to the medium. Monatin was not detected in any of the negative control cell extracts. *In vitro* assays for monatin were performed in duplicate with 4.4 mg/mL of total protein (about double what is typically used for *E. coli* cell extracts) using the optimized assay described in Example 6. Other assays were performed with the addition of either 32 µg/mL *C. testosteroni* ProA aldolase or 400 µg/mL AspC aminotransferase, to determine which enzyme was limiting in the cell extract. Negative controls were performed with no addition of enzyme, or the addition of only AspC aminotransferase (the aldol condensation can occur to some extent without enzyme). Positive controls were performed with partially pure enzymes (30-40%), using 16 µg/mL aldolase and 400 µg/mL aminotransferase.

In vitro results were analyzed by SRM. The analysis of cell extracts showed that tryptophan was effectively transported into the cells when it was added to the medium post-induction, resulting in tryptophan levels two orders of magnitude higher than those in which no additional tryptophan was added. The results for *in vitro* monatin analysis are shown in Table 5 (numbers indicate ng/mL).

Table 5

Monatin production with yeast cell extracts

	aspC construct	+ aldolase	+ AspC	two-gene construct	+ aldolase	+ AspC
repressed (glucose medium)	0	888.3	173.5	0	465.2	829
24 hr induced	0	2832.8	642.4	0	1375.6	9146.6
69 hr induced	0	4937.3	340.3	71.9	1652.8	23693.5
69 hr + subs.	0	556.9	659.1	21.9	755.6	16688.2
+ control (purified enzymes)	21853			21853		
-control (no enzymes)	0		254.3	0		254.3

Positive results were obtained with the full two-gene construct cell extracts with and without substrate added to the growth medium. These results, in comparison to the positive controls, indicate that the enzymes were expressed at levels of close to 1% of the total protein in yeast. The amount of monatin produced when the cell extract of the *aspC* construct (with truncated *proA*) was assayed with aldolase was significantly greater than when cell extracts were assayed alone, and indicates that the recombinant AspC aminotransferase comprises approximately 1-2% of the yeast total protein. The cell extracts of uninduced cultures had a small amount of activity when assayed with aldolase due to the presence of native aminotransferases in the cells. When assayed with AspC aminotransferase, the activity of the extracts from uninduced cells increased to the amount of monatin produced by the negative control with AspC (ca. 200 ng/mL). In contrast, the activity observed when assaying the two gene construct cell extract increases more when aminotransferase is supplemented than when aldolase is added. Since both genes should be expressed at the same level, this indicates that the amount of monatin produced is maximized when the level of aminotransferase is higher than that of aldolase, in agreement with results shown in Example 6.

The addition of pyruvate and tryptophan not only inhibits cellular growth, but apparently inhibits protein expression as well. The addition of the pESC-Trp plasmid can be used to correct for tryptophan auxotrophy of the YPH500 host cells, to provide a means of supplying tryptophan with fewer effects on growth, expression, and secretion.

EXAMPLE 13

Improvement of Enzymatic Processes using Coupled Reactions

In theory, if no side reactions or degradation of substrates or intermediates occurs, the maximum amount of product formed from the enzymatic reaction illustrated in FIG. 1 is directly proportional to the equilibrium constants of each reaction, and the concentrations of tryptophan and pyruvate. Tryptophan is not a highly soluble substrate, and concentrations of pyruvate greater than 200 mM appear to have a negative effect on the yield (see Example 6).

Ideally, the concentration of monatin is maximized with respect to substrates, in order to decrease the cost of separation. Physical separations can be performed such that the monatin is removed from the reaction mixture, preventing the reverse reactions from occurring. The raw materials and catalysts can then be regenerated. Due to the similarity of monatin in size, charge, and hydrophobicity to several of the reagents and intermediates, physical separations will be difficult unless there is a high amount of affinity for monatin (such as an affinity chromatography technique). However, the monatin reactions can be coupled to other reactions such that the equilibrium of the system is shifted toward monatin production. The following are examples of processes for improving the yield of monatin obtained from tryptophan or indole-3-pyruvate.

Coupled reactions using oxaloacetate decarboxylase (EC 4.1.1.3)

FIG. 11 is an illustration of the reaction. Tryptophan oxidase and catalase are utilized to drive the reaction in the direction of indole-3-pyruvate production. Catalase is used in excess such that hydrogen peroxide is not available to react in the reverse direction or to damage the enzymes or intermediates. Oxygen is regenerated during the catalase reaction. Alternatively, indole-3-pyruvate can be used as the substrate.

Aspartate is used as the amino donor for the amination of MP, and an aspartate aminotransferase is utilized. Ideally, an aminotransferase that has a low specificity for the tryptophan/indole-3-pyruvate reaction in comparison to the MP to monatin reaction is used so that the aspartate is not utilized to reaminate the indole-3-pyruvate. Oxaloacetate decarboxylase (from *Pseudomonas sp.*) can be added to convert the oxaloacetate to pyruvate and carbon dioxide. Since CO_2 is volatile, it is not available for reaction with the enzymes, decreasing or even preventing the reverse reactions. The pyruvate produced in this step can also be utilized in the aldol condensation reaction. Other decarboxylase enzymes can be used, and homologs are known to exist in *Actinobacillus actinomycetemcomitans, Aquifex*

aeolicus, Archaeoglobus fulgidus, Azotobacter vinelandii, Bacteroides fragilis, several *Bordetella* species, *Campylobacter jejuni, Chlorobium tepidum, Chloroflexus aurantiacus, Enterococcus faecalis, Fusobacterium nucleatum, Klebsiella pneumoniae, Legionella pneumophila, Magnetococcus MC-1, Mannheimia haemolytica, Methylobacillus flagellatus KT, Pasteurella multocida* Pm70, *Petrotoga miotherma, Porphyromonas gingivalis*, several *Pseudomonas* species, several *Pyrococcus* species, *Rhodococcus*, several *Salmonella* species, several *Streptococcus* species, *Thermochromatium tepidum, Thermotoga maritima, Treponema pallidum*, and several *Vibrio* species.

Tryptophan aminotransferase assays were performed with the aspartate aminotransferase (AspC) from *E. coli*, the tyrosine aminotransferase (TyrB) from *E. coli*, the broad substrate aminotransferase (BSAT) from *L. major*, and the two commercially available porcine glutamate-oxaloacetate aminotransferases as described in Example 1. Both oxaloacetate and alpha-ketoglutarate were tested as the amino acceptor. The ratio of activity using monatin (Example 7) versus activity using tryptophan was compared, to determine which enzyme had the highest specificity for the monatin aminotransferase reaction. These results indicated that the enzyme with the highest specificity for the monatin reaction verses the tryptophan reaction is the Porcine type II-A glutamate-oxaloacetate aminotransferase, GOAT (Sigma G7005). This specificity was independent of which amino acceptor was utilized. Therefore, this enzyme was used in the coupled reactions with oxaloacetate decarboxylase.

A typical reaction starting from indole-3-pyruvate included (final concentrations) 50 mM Tris-Cl pH 7.3, 6 mM indole-3-pyruvate, 6 mM sodium pyruvate, 6 mM aspartate, 0.05 mM PLP, 3 mM potassium phosphate, 3 mM $MgCl_2$, 25 μg/mL aminotransferase, 50 μg/mL *C. testosteroni* ProA aldolase, and 3 Units/mL of decarboxylase (Sigma O4878). The reactions were allowed to proceed for 1 hour at 26°C. In some cases, the decarboxylase was omitted or the aspartate was substituted with alpha-ketoglutarate (as negative controls). The aminotransferase enzymes described above were also tested in place of the GOAT to confirm earlier specificity experiments. Samples were filtered and analyzed by LC/MS as described in Example 10. The results demonstrate that the GOAT enzyme produced the highest amount of monatin per mg of protein, with the least amount of tryptophan produced as a byproduct. In addition, there was a 2-3 fold benefit from having the decarboxylase enzyme added. The

E. coli AspC enzyme also produced large amounts of monatin in comparison to the other aminotransferases.

Monatin production was increased by: 1) periodically adding 2 mM additions of indole-pyruvate, pyruvate, and aspartate (every half hour to hour), 2) performing the reactions in an anaerobic environment or with degassed buffers, 3) allowing the reactions to proceed overnight, and 4) using freshly prepared decarboxylase that has not been freeze-thawed multiple times. The decarboxylase was inhibited by concentrations of pyruvate greater than 12 mM. At concentrations of indole-3-pyruvate higher than 4 mM, side reactions with indole-3-pyruvate were hastened. The amount of indole-3-pyruvate used in the reaction could be increased if the amount of aldolase was also increased. High levels of phosphate (50 mM) and aspartate (50 mM) were found to be inhibitory to the decarboxylase enzyme. The amount of decarboxylase enzyme added could be reduced to 0.5 U/mL with no decrease in monatin production in a one hour reaction. The amount of monatin produced increased when the temperature was increased from 26°C to 30°C and from 30°C to 37°C; however, at 37°C the side reactions of indole-3-pyruvate were also hastened. The amount of monatin produced increased with increasing pH from 7 to 7.3, and was relatively stable from pH 7.3-8.3.

A typical reaction starting with tryptophan included (final concentrations) 50 mM Tris-Cl pH 7.3, 20 mM tryptophan, 6 mM aspartate, 6 mM sodium pyruvate, 0.05 mM PLP, 3 mM potassium phosphate, 3 mM $MgCl_2$, 25 μg/mL aminotransferase, 50 μg/mL *C. testosteroni* ProA aldolase, 4 Units/mL of decarboxylase, 5-200 mU/mL L-amino acid oxidase (Sigma A-2805), 168 U/mL catalase (Sigma C-3515), and 0.008 mg FAD. Reactions were carried out for 30 minutes at 30°C. Improvement was observed with the addition of decarboxylase. The greatest amount of monatin was produced when 50 mU/mL of oxidase was used. Improvements were similar to those observed when indole-3-pyruvate was used as the substrate. In addition, the amount of monatin produced increased when 1) the tryptophan level was low (i.e., below the K_m of the aminotransferase enzyme and therefore unable to compete with MP in the active site), and 2) the ratio of oxidase to aldolase and aminotransferase was maintained at a level such that indole-3-pyruvate could not accumulate.

Whether starting with either indole-3-pyruvate or tryptophan, the amount of monatin produced in assays with incubation times of 1-2 hours increased when 2-4 times the amounts of all the enzymes were used while maintaining the same enzyme ratio. Using either substrate, concentrations of approximately 1 mg/mL of monatin were achieved. The amount of tryptophan produced if starting from indole-pyruvate was typically less than 20% of the amount of product, which shows the benefit of utilizing coupled reactions. With further optimization and control of the concentrations of intermediates and side reactions, the productivity and yield can be improved greatly.

Coupled reactions using lysine epsilon aminotransferase (EC 2.6.1.36)

Lysine epsilon aminotransferase (L-Lysine 6-transaminase) is found in several organisms, including *Rhodococcus, Mycobacterium, Streptomyces, Nocardia, Flavobacterium, Candida utilis*, and *Streptomyces*. It is utilized by organisms as the first step in the production of some beta-lactam antibiotics (Rius and Demain, *J. Microbiol. Biotech.*, 7:95-100, 1997). This enzyme converts lysine to L-2-aminoadipate 6-semialdehyde (allysine), by a PLP-mediated transamination of the C-6 of lysine, utilizing alpha-ketoglutarate as the amino acceptor. Allysine is unstable and spontaneously undergoes an intramolecular dehydration to form 1-piperideine 6-carboxylate, a cyclic molecule. This effectively inhibits any reverse reaction from occurring. The reaction scheme is depicted in FIG. 12. An alternative enzyme, lysine-pyruvate 6-transaminase (EC 2.6.1.71), can also be used.

A typical reaction contained in 1 mL: 50 mM Tris-HCl pH 7.3, 20 mM indole-3-pyruvate, 0.05 mM PLP, 6 mM potassium phosphate pH 8, 2-50 mM sodium pyruvate, 1.5 mM $MgCl_2$, 50 mM lysine, 100 μg aminotransferase (lysine epsilon aminotransferase LAT-101, BioCatalytics Pasadena, CA), and 200 μg *C. testosteroni* ProA aldolase. The amount of monatin produced increased with increasing concentrations of pyruvate. The maximum amount using these reaction conditions (at 50 mM pyruvate) was 10-fold less than what was observed with coupled reactions using oxaloacetate decarboxylase (approximately 0.1 mg/mL).

A peak with $[M+H]^+ = 293$ eluted at the expected time for monatin and the mass spectrum contained several of the same fragments observed with other enzymatic processes. A second peak with the correct mass to charge ratio (293) eluted slightly earlier than what is typically

observed for the S,S monatin produced in Example 6, and may indicate the presence of another stereoisomer of monatin. Very little tryptophan was produced by this enzyme. However, there is likely some activity on pyruvate (producing alanine as a byproduct). Also, the enzyme is known to be unstable. Improvements can be made by performing directed evolution experiments to increase stability, reduce the activity with pyruvate, and increase the activity with MP. These reactions can also be coupled to L-amino acid oxidase/catalase as described above.

Other coupled reactions

Another coupling reaction that can improve monatin yield from tryptophan or indole-pyruvate is shown in FIG. 13. Formate dehydrogenase (EC 1.2.1.2 or 1.2.1.43) is a common enzyme. Some formate dehydrogenases require NADH while others can utilize NADPH. Glutamate dehydrogenase catalyzed the interconversion between the monatin precursor and monatin in previous examples, using ammonium based buffers. The presence of ammonium formate and formate dehydrogenase is an efficient system for regeneration of cofactors, and the production of carbon dioxide is an efficient way to decrease the rate of the reverse reactions (Bommarius *et al.*, *Biocatalysis* 10:37, 1994 and Galkin *et al. Appl. Environ. Microbiol.* 63:4651-6, 1997). In addition, large amounts of ammonium formate can be dissolved in the reaction buffer. The yield of monatin produced by glutamate dehydrogenase reactions (or similar reductive aminations) can be improved by the addition of formate dehydrogenase and ammonium formate.

Other processes can be used to drive the equilibrium toward monatin production. For instance, if aminopropane is utilized as the amino acid donor in the conversion of MP to monatin with an omega-amino acid aminotransferase (EC 2.6.1.18) such as those described by in US patents 5,360,724 and 5,300,437, one of the resulting products would be acetone, a more volatile product than the substrate, aminopropane. The temperature can be raised periodically for short periods to flash off the acetone, thereby alleviating equilibrium. Acetone has a boiling point of 47°C, a temperature not likely to degrade the intermediates if used for short periods of time. Most aminotransferases that have activity on alpha-ketoglutarate also have activity on the monatin precursor. Similarly, if a glyoxylate/aromatic acid aminotransferase (EC 2.6.1.60) is used with glycine as the amino donor, glyoxylate is

produced which is relatively unstable and has a highly reduced boiling point in comparison to glycine.

EXAMPLE 14: Dose Response Curve

Solutions of monatin (mixture of approximately 96% of the 2R,4R/2S, 4S enantiometric pair and 4% of the 2R,4S/2S,4R enantiometric pair—also called "racemic mix" of monatin") at 15, 30, 45, 60, 75 and 90 ppm were prepared in a pH 3.2 model soft drink system that contained 0.14% (w/v) citric acid and 0.04% (w/v) sodium citrate. The sweetness of monatin relative to sucrose was determined using the sweetness estimation methodology described below. All assessments were carried out in duplicate by a panel (n = 6-8) of trained panelists experienced in this sweetness determination procedure. All samples were served at a temperature of 20°C ± 1°C.

Monatin solutions were coded and presented individually to panelists, in random order. Sucrose reference standards, ranging from 2.0 - 11.0% (w/v) sucrose, increasing in steps of 0.5% (w/v) sucrose also were provided. Panelists were asked to estimate sweetness by comparing the sweetness of the test solution to the sucrose standards. This was carried out by taking 3 sips of the test solution, followed by a sip of water, followed by 3 sips of sucrose standard followed by a sip of water, etc. Panelists were encouraged to estimate the sweetness to one decimal place, e.g., 6.8, 8.5. A five minute rest period was imposed between evaluating the test solutions. Panelists also were asked to rinse well and eat a cracker to reduce any potential carry over effects. The sucrose equivalence values (SEVs) and standard deviations are summarized in Table 6.

The blends were all judged to exhibit rapid onset to sweetness and sweetness build to maximum intensity. The decay of sweetness also was rapid. Most of the mixtures were judged less fruity than sucrose, except the monatin/glucose blend. A slight lingering sweetness aftertaste was noted, very slight bitter/metallic notes. No licorice or cooling aftertaste was noted.

TABLE 6

Monatin Dose Response Data

Monatin Conc. (ppm)	SEV (%; w/v)	Standard Deviation
15	3.6	±0.7
30	4.9	±0.5
45	7.1	±0.6
60	8.5	±0.5
75	9.8	±0.5
90	10.5	±0.6

EXAMPLE 15: Blending of Monatin with Carbohydrate Sweeteners

Blends of monatin (as described in Example 14) with sucrose, HFCS (55% fructose), and glucose syrup (63 dextrose equivalents, DE) equisweet to 10.0% (w/v) sucrose were prepared. For each carbohydrate sweetener, the monatin:sweetener ratio was adjusted so that monatin delivered 25, 50, and 75% of the total sweetness. Sweetness parity to 10.0 % (w/v) sucrose was determined using the sweetness estimation method described in Example 14. As in Example 14, all assessments were carried out in the pH 3.2 model soft drink system, using 6-8 panelists, each tasting in duplicate. Results are presented as Tables 7-9. Monatin compared similarly to sucralose, with a slight delay in onset of sweetness.

TABLE 7

Equisweet Blends of Monatin and Sucrose

Sweetness Contribution of Monatin (%)	Sucrose Conc. (%; w/v)	Monatin Conc. (ppm)	Effective Relative Sweetness Intensity (x sucrose) of Monatin
25	7.5	12.3	2000
50	5.0	30.8	1600
75	2.5	50.3	1500

TABLE 8

Equisweet Blends of Monatin and HFCS

Sweetness Contribution of Monatin (%)	HFCS Conc. (%; w/v solids)	Monatin Conc. (ppm)
25	7.8	12.3
50	4.7	30.8
75	2.7	50.3

TABLE 9

Equisweet Blends of Monatin and Glucose Syrup

Sweetness Contribution of Monatin (%)	Glucose Syrup Conc. (%; w/v solids)	Monatin Conc. (ppm)
25	16.4	12.3
50	10.4	30.8
75	5.4	50.3

The quality of equisweet monatin/carbohydrate (50:50) blends then was assessed relative to sucrose by a small panel of trained assessors. This evaluation was carried out "double blind." The sucrose-sweetened system was identified as the control and all other products randomly coded. Panelists were asked to assess the randomly coded sample relative to the control for the following attributes: Sweetness Profile: Onset, build and decay; Flavor Profile: Acidity, bitterness and other characteristics; Mouthfeel; and Aftertaste. Panelists also were asked to assign a score (1; poor – 5; good) for the quality of the sweetener system. A summary of the comments made and scores given is presented as Table 10.

TABLE 10

Taste Profiling of Monatin/Carbohydrate Blends

Sweetener System	Sweetness Profile	Flavor Profile	Mouthfeel	Aftertaste	Average Score
Sucrose	Fast onset and build to peak intensity. Quick and clean decay.	Pleasant, citrus acidity. No bitterness detectable.	Full, syrupy and warm.	Slight lingering sweetness, not sickly in nature. No off flavors detectable.	4.0
Sucrose/ Monatin	Fast onset and build. Overall profile quite flat in nature. Quite quick and clean to decay.	Less fruity and citrusy than sucrose. Slight bitterness detectable.	Syrupy, but slightly thinner than sucrose.	Slight lingering sweetness detectable. Slightly bitter and acidic.	3.4
HFCS/ Monatin	Fast onset and build to maximum intensity. Quite quick to decay, some lingering sweetness. Very slightly sickly in nature.	Less fruity and citrusy than sucrose. Slight candy floss note detectable. Slightly bitter.	Thinner mouthfeel than sucrose. Some drying once sweetness disappears.	Slight lingering sweetness detectable. Some bitter/metallic notes perceived. Quite drying, empty aftertaste.	2.9
Glucose Syrup/ Monatin	Quick onset and build to maximum intensity, slightly slower than sucrose. Quick and clean to decay.	Very similar to sucrose.	Full, sugary and warm. Similar to sucrose.	Slight sweetness detectable.	3.2

EXAMPLE 16: Time Intensity Profile of Monatin in a Soft Drink System

Solutions of 80 ppm monatin (racemic mix of monatin described in Example 14), 10.0% (w/v) sucrose and 200 ppm sucralose were prepared in the pH 3.2 model soft drink system described in Example 14. The time intensity profile of these solutions then was assessed using the following procedure. Six panelists were included in the study. These panelists were screened for their general sensory acuity and selected for their sensitivity to sweetness intensity and sweetness quality differences. All were experienced in methods of sweetener assessment and had received special training in time intensity evaluations. Training sessions were carried out initially to familiarize the panel with the method of evaluation and scoring the samples over time using a computerized data entry system.

Samples of each solution (13 mL) were coded and presented individually to panelists, in random order. For each panelist, immediately after swallowing, the computer recorded timed intensity readings on the scale of 0-100 each second, up to 60 seconds. Each solution was evaluated in duplicate. The results of the time intensity evaluation are summarized as Table 11.

TABLE 11

Time Intensity Study Results

	Sucrose	Monatin	Sucralose
Intensity of Maximum Sweetness (unit)	64.1	66.6	64.6
Time to Maximum Sweetness (s)	8.0	9.0	8.0
Time to Half Maximum Sweetness (s)	2.3	2.4	2.6
Time for Sweetness to Decline to Half Maximum Value (s)	24.9	34.2	33.1
Rate of Onset (unit/s)	17.9	14.9	16.0
Rate of Decline (unit/s)	2.3	2.2	2.1
Area Under Curve (unit x s)	116.9	117.3	119.7

These results indicate that the temporal taste attributes of monatin are comparable to sucrose, which is indicative of a high quality sweetener. Additionally, monatin compares favorably to sucralose, a commonly used high intensity sweetener.

EXAMPLE 17: Preparation of Cola and Lemon/lime Beverages Containing Monatin

Cola and lemon/lime beverages were prepared using the following formulations and sweetened with sucrose, HFCS (55% fructose), aspartame, sucralose, monatin (racemic mix described in Example 14), monatin/sucrose, or monatin/HFCS. One part of syrup was added to 5.5 parts carbonated water and evaluated.

Lemon/Lime Syrup Formulation:

Ingredient	% wt/vol
citric acid	2.400
sodium citrate	0.500
sodium benzoate	0.106
Flavor	0.450 (Lemon/Lime Flavor 730301-H ex. Givaudan Roure)
Sweeteners	see below
Water to	100.000

Cola Syrup Formulation:

Ingredient	% wt/vol
Phosphoric Acid	0.650 (75% solution)
citric acid	0.066
sodium citrate	0.300
sodium benzoate	0.106
Cola Flavor A	1.100 (A01161 ex. Givaudan Roure)
Cola Flavor B	1.100 (B01162 ex. Givaudan Roure)
Sweeteners	see below
Water to	100.000

Sweetener concentration in lemon/lime or cola carbonate:

sucrose	10%
HFCS (55% Fructose)	10% (solids)
Aspartame	500 ppm
Sucralose	200 ppm
Monatin	67 ppm (in lemon/lime); 80 ppm (in cola)
Monatin/sucrose	30.8 ppm/5.0%
Monatin/HFCS	30.8 ppm/5.0% (solids)

Assessments were carried out 'double blind' by a panel of trained tasters. The sucrose-sweetened product was identified as the control and all other products randomly coded. Panelists were asked to assess the randomly coded sample relative to the control for the following attributes:

Flavor Profile:	Acidity
	Bitterness
	Other Characteristics
Sweetness Profile:	Onset
	Build
	Intensity
	Decay
Mouthfeel	
Aftertaste	

Panelists also were asked assign a score (1; poor – 5; good) for the quality of the sweetener system. A summary of the comments generated together with the average score awarded is presented in Tables 12 and 13 for lemon/lime carbonates and colas, respectively. In the lemon/lime flavor, monatin was comparable in flavor to aspartame. Blends of monatin/carbohydrate rated higher. In the cola, monatin was similar to aspartame.

TABLE 12

Taste Profiling Lemon/Lime Carbonates

Sweetener System	Flavor Profile	Sweetness Profile	Mouthfeel	Aftertaste	Average Score
Sucrose	Soft, balanced lemon/lime flavor. Slightly lacking freshness. More lime detectable than lemon.	Slight delay in sweetness onset but rapid build to peak intensity. Quick decay.	Warm, quite full and syrupy – particularly towards the end.	Slight bitterness and some astringency. Some sweetness but not sickly or lingering.	4.8
HFCS	Quite fruity and zesty. Slightly more acidic than control. More lime detectable than lemon.	Clean profile. Fast onset, quick to build to peak intensity, quick to decay.	Thinner than control. Slightly watery.	Slightly bitter. Not quite a clean as the control.	3.6
Aspartame	Slightly lacking upfront. Quite similar to control later in profile.	Sweetness onset quite quick. Overall peak quite flat. Some lingering sweetness detectable.	Slightly thinner and colder than control.	Quite clean but some lingering sweetness. Slight "aspirin" like notes detectable.	4.0
Sucralose	Zesty and refreshing flavor. Some oiliness also detectable.	Delayed onset, but builds relatively quickly. Some sweetness detectable at the back of the throat.	Similar mouthfeel to control.	Slight licorice note detectable in aftertaste. Some bitterness also detectable.	4.1

TABLE 12 continued: Taste Profiling Lemon/Lime Carbonates

Sweetener System	Flavor Profile	Sweetness Profile	Mouthfeel	Aftertaste	Average Score
Monatin	Softer flavor than control. Less depth and less acidic.	Slight delay in onset – slightly greater than control. Flat sweetness profile, rather than building to a peak. Slightly slower than control to decay.	Slightly less mouthfeel than control. But quite full and syrupy.	Quite clean, slight lingering sweetness detectable. Some bitterness and metallic notes also detectable.	4.0
Monatin/ Sucrose	Slightly brighter and fruitier than control. Zesty flavor. Quite refreshing.	Clean and rounded profile. Quick onset, build and decay.	Full and syrupy. Slightly colder than control.	Clean aftertaste. Some flavor and acidity detectable in aftertaste. Slightly sweet, but not overpowering or sickly in nature.	5.0
Monatin/ HFCS	Slightly less flavor than control. Slightly less acidity.	Slight delay in onset. Broad, rounded peak. Good rate of decay.	Quite full, syrupy and warm. Slightly less than control.	Slightly sweet, but quite clean. Not sickly in nature.	4.4

TABLE 13

Taste Profiling of Colas

Sweetener System	Flavor Profile	Sweetness Profile	Mouthfeel	Aftertaste	Average Score
Sucrose	Sweet, rounded, warm cola flavor. Quite spicy, citrus and lemon in nature. Slightly acidic towards the end.	Very slight delay in sweetness onset. Rapid build to a rounded peak. Quick to decay.	Quite warm, full and syrupy mouthfeel.	Quite clean and balanced. Slight sweetness. Slightly bitter, some flavor and acidity also detectable.	4.5
HFCS	Sweet, slightly spicy. Softer with less depth of flavor than control, particularly upfront.	Slight delay in sweetness onset. Flatter sweetness profile. Quite quick to decay.	Quite full but colder and slightly thinner than the control.	Some sweetness detectable but not sickly in nature. Slightly bitter and acidic.	3.3
Aspartame	Sweet flavor, flatter upfront than control. Fewer brown/caramel and spice notes but more lemon notes detectable.	Delayed sweetness onset. Slightly slower build to peak than control and some lingering sweetness. But, overall quite a rounded peak.	Thinner mouthfeel than control, but still quite warm.	Lingering sweetness detectable, slightly sickly in nature. More bitter than control.	3.3
Sucralose	Sweet flavor, slightly browner than control upfront. Then becomes more acidic and lemony towards the end.	Some delayed to sweetness onset. Slightly slower than control to build, appears to build through profile.	Slightly thinner than control but still quite full and syrupy.	Some sweetness detectable in aftertaste. Slightly sickly in nature. Flavor detectable, carried through by sweetness.	3.8

TABLE 13 continued: Taste Profiling of Colas

Sweetener System	Flavor Profile	Sweetness Profile	Mouthfeel	Aftertaste	Average Score
Monatin	Slightly flatter than control. More acidic and citrusy in nature. Less warm and caramellic.	Delayed onset, but builds quite quickly. Overall, flatter profile than control. Slower to decay than control, some lingering sweetness detectable.	Thinner than control. Slightly colder and more watery upfront.	Some lingering sweetness detectable – sickly in nature. Some bitterness also detectable.	2.9
Monatin/ Sucrose	Less cola notes than control. Flatter and less spicy. Flavor more citrus/lemony in nature, particularly towards end.	Very slight delay in onset. Builds quite quickly. Relatively quick decay, no lingering sweetness. Flatter profile rather than building to a peak.	Slightly colder than control. Quite full and syrupy.	Slightly more bitter than the control. Slight sweetness but less flavor.	3.0
Monatin/ HFCS	Full, warm and spicy upfront. Slightly empty towards end, more citrus/lemon notes than control.	Quick onset, and builds quite quickly to peak sweetness. Slightly slow to decay. Some lingering sweetness detectable.	Slightly thinner than control.	Some sweetness detectable. Slightly sickly in nature. Some flavor, bitterness and acidity also detectable.	3.3

Discussion

The monatin used in this example elicited a clean, sweet taste profile, essentially free from bitterness, cooling and licorice flavors often observed in natural high intensity sweeteners. The blend of monatin stereoisomers used in this example produced a smooth, regular dose response curve with a relative sweetness intensity 1250x sweeter than sucrose at 10.0% (w/v) SEV.

The results of the time intensity study showed that the monatin exhibited a time/sweetness intensity profile broadly similar to that of sucrose and sucralose. In comparison with sucrose, monatin took slightly longer to achieve maximum intensity and exhibited a slower rate of decay, with a higher perceived sweetness at the end of the evaluation (60s). However, the differences observed were not statistically significant.

When blended with carbohydrate sweeteners, the monatin delivered a sweetness intensity 1500 – 2000x sucrose. The resulting blends produced a very good quality sweetness and flavor profile. Little delay in sweetness onset was observed with only a low level of lingering sweetness detectable. Blends of monatin and carbohydrate sweeteners can be used, for example, to prepare mid-calorie beverages.

The evaluated monatin performed well both as a sole sweetener and when blended with carbohydrate sweeteners. In lemon/lime carbonates the product solely sweetened with monatin had a very similar taste profile to both the aspartame and sucralose sweetened drinks. The monatin/sucrose drink was particularly good and was actually judged more acceptable than the sucrose control product. It is expected that monatin will enhance the lemon/lime flavor in blends with other carbohydrate sweeteners. In the cola system, blending monatin with HFCS produced a drink as acceptable as the HFCS control.

Example 18: Sensory stability of monatin in water

The sensory stability of monatin (racemic mix described in Example 14) in water (8% SEV) was studied after storage at room temperature for 0 to 6 hours. The SEV was monitored (as described above in Example 14) at either 0-1 hours or 5-6 hours after

preparing a monatin solution. There was no detectable loss of monatin SEV after 6 hours in room temperature; these data were corroborated by analytical studies using LC/MS (e.g., no lactonization was observed).

EXAMPLE 19: Preparation of a Malted Beverage Premix

A malted beverage premix is prepared using the ingredients listed in Table 14.

TABLE 14

Ingredient	% (by weight)
Malt extract	31-35
Skimmed milk powder	10-12
Cocoa	5-10
Monatin	0.001-0.46
Fats	8-9
Minerals and vitamin	0.5- 1
Diluent	as needed

EXAMPLE 20: Preparation of a Chocolate Flavored Beverage Premix

A chocolate flavored beverage premix is prepared using the ingredients listed in Table 15. Non-dairy creamers can include vegetable oil, thickening agents, lecithin, protein, vitamins, minerals, emulsifiers (such as lecithin, DATEM and mono- and diglycerides) and bulking agents (e.g., corn syrup solids, low-calorie bulking agents).

TABLE 15

Ingredient	% (by weight)
Cocoa powder	3-13
Caramel powder	3-5
Malt extract	10-20
Monatin	0.015-1
Flavor enhancer/salt	0.25-1

Non-dairy creamer	10-32
Diluent	as needed

EXAMPLE 21: Preparation of an Orange Flavored Beverage Premix

An orange flavored beverage premix is prepared using the ingredients listed in Table 16.

TABLE 16

Ingredient	% (by weight)
Whey Protein Concentrate	60-70
Fructose	20-25
Dry Sweet Whey	8-10
Citric Acid, Anhydrous	3-7
Orange Flavor	0.5-1
Vitamin/Mineral Premix	0.10-0.15
Monatin	S,S 0.06-0.35, R,R 0.006-0.01 or a mixture
Artificial colors	0.006-0.010

An orange beverage can be made by mixing approximately 1 oz. of the dry mix in 8 oz. water, then stirring or shaking until fully hydrated. Thus, the final ready-to-drink beverage has from about 66 to about 440 ppm S,S monatin, from about 6 to about 13 ppm R,R, or a mixture thereof.

EXAMPLE 22: Preparation of Lemonade Using a Monatin Sweetener

One may prepare convenient single-serving packets of sweetener comprising monatin, where the sweetener is formulated to provide a sweetness comparable to that in 2 teaspoons (~8 grams) of granulated sugar. Because S,S is 50-200 times sweeter than sucrose, 40-160 mg of S,S monatin delivers a sweetness comparable to that in 8 grams of granulated sugar. Thus, for example, allowing for +/– 25% sweetness

optimization, single-serving packet 1 gram formulations of monatin may comprise approximately 40-200 mg of S,S monatin.

Likewise, because R,R is 2000-2400 times sweeter than sucrose, 3.3-4.0 mg of R,R monatin delivers a sweetness comparable to that in 8 grams of sugar. Thus, in another embodiment, allowing for +/– 25% sweetness optimization, single-serving packet 1 gram formulations of monatin may comprise approximately 3.3-5.0 mg of R,R monatin. In another embodiment, packet formulations may comprise 40-200 mg of S,S monatin, 3.3-5.0 mg of R,R monatin or a combination thereof in the same or lesser amounts per gram total weight, to provide a sweetness comparable to that in 2 teaspoons of granulated sugar.

To make lemonade, mix 2 tablespoons of lemon juice and 3 packets (3 g) of a monatin packet formulation with ¾ cup of water in a tall glass until dissolved. Add ice. The monatin-sweetened lemonade will be nearly equivalent in sweetness and equally preferred to the lemonade sweetened with 6 teaspoons (24 g) sucrose and will have significantly fewer calories (about 0 Calories versus 96 Calories).

EXAMPLE 23: Evaluation of R,R Monatin-Containing Sweeteners In Coffee and Iced Tea.

Monatin sweetener formulations, comprising R,R monatin or R,R monatin/erythritol combinations, were assessed relative to other known sweeteners (aspartame and sucralose) in coffee and iced tea. The key sensory parameters assessed included sweetness quality, aftertaste, bitter taste and its aftertaste. Qualitative evaluation was carried out.

Product Formulations

(i) Coffee

Standard coffee was used in which to evaluate sweetener performance (Table 17).

Table 17. Coffee formulation

Ingredient	Supplier	Concentration (%; w/v)	g/700mL
Classic Roast Coffee	Folger®	5.41	37.87
Water		94.59	662.13

Sweeteners were added to coffee at the following concentrations:

Aspartame	0.025% (w/v)
Sucralose	0.0082% (w/v)
R,R monatin	0.0020, 0.0025, 0.0030% (w/v) plus 1 g maltodextrin
R,R monatin/erythritol	0.0020, 0.0025, 0.0030% (w/v) plus 1 g erythritol

(ii) Iced Tea

An ice tea formulation was developed to evaluate sweetener performance (Table 18).

Table 18. Iced Tea formulation

Ingredient	Supplier	Concentration (%; w/v)
Citric acid		0.200
Sodium citrate		0.020
Tea extract 'Assam' 285002	Plantextrakt	0.150
Natural black tea flavor extract 31108304010000	Rudolph Wild	0.050
Sodium benzoate (20% w/w)		0.075
Sweetener		As required
Water		To volume

Sweeteners were added to tea at the following concentrations:

Aspartame	0.0450% (w/v)
Sucralose	0.0170% (w/v)
R,R monatin	0.0030, 0.0035, 0.0040% (w/v) plus 1 g maltodextrin
R,R, monatin/erythritol	0.0030, 0.0035, 0.0040% (w/v) plus 1 g erythritol

Sensory Evaluation

The evaluation of these coffee and tea drinks was carried out by a panel (n = 6) of experienced sensory evaluators who evaluated the coffee products on one tasting occasion and the tea products on a subsequent occasion. The results of these evaluations are summarized in Table 19.

Table 19. Sensory evaluation of coffee and tea (200 mL serving size)

Product	Sweetener/concentration	Comments
Coffee	Aspartame/250ppm	Balanced sweetness profile. Very low level of bitterness, probably due to inhibition by APM. Flat, even coffee flavor delivery. Typical APM aftertaste that is perceived at the back of the tongue.
	Sucralose/82ppm	Slow sweetness onset allows stronger coffee notes to be perceived. Bitter coffee notes quite clearly apparent in the aftertaste, although balanced somewhat by the lingering sweet character of sucralose.
	Monatin (25ppm) + Maltodextrin (1 g) (0.5%)	Balanced sweetness profile. Clear coffee flavor in the aftertaste. Stronger coffee flavor overall than with either of the other sweeteners, although this may be (at least in part) due to the limited bitterness inhibiting capacity of monatin.
	Monatin (25ppm) + Erythritol (1 g) (0.5%)	More coffee flavor in monatin sample. Sweetness is less delayed with monatin/erythritol combination than with monatin/maltodextrin. Erythritol smoothes out the coffee flavor and makes the sweetness onset a little faster.
Iced Tea	Aspartame/450ppm	Good temporal characteristics although the typical aspartame flavor is clearly apparent. Balanced, though quite subtle tea flavor. No evidence of flavor enhancement.
	Sucralose/170ppm	Delay in sweetness onset means first impressions are of acidity. Product flavor and overall impression somewhat out of balance because of sweetness profile not matching acidity or flavor profiles.
	Monatin (40ppm) + Maltodextrin (1 g) (0.5%)	Sweetness and flavor profiles very balanced. The lemon flavor notes are clearly enhanced over those of the other sweeteners.
	Monatin (40ppm) + Erythritol (1 g) (0.5%)	Sweetness and flavor profiles balanced. Lemon flavor notes even more enhanced than monatin/maltodextrin alone. The astringency in the aftertaste is greatly reduced/eliminated.

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Discussion

Monatin delivered unexpected performance benefits, including clear sensory benefits, in sweetener formulations. When monatin was added to coffee, a clear increase in the level of coffee flavor was perceived. This benefit was further enhanced through addition of low concentrations of erythritol, which were able to balance and round the flavor and to speed up sweetness onset times. In iced-tea, and particularly acidified acid tea, monatin enhanced the lemon flavor notes. Again, erythritol blending with monatin conferred additional flavor benefits.

Monatin delivers improved sensory properties (e.g., less aftertaste, less off-taste, no flavor masking) in commonly consumed beverages such as tea and coffee. Monatin sweetened coffee contains close to 0 Calories, as compared to 32 Calories in coffee sweetened with 2 teaspoons (~8 g) of sucrose.

It is expected that in beverage compositions, monatin exhibits enhancement of all citrus flavors, as well as provides a more favorable time/intensity profile for sweetness, as compared to aspartame or sucralose. It is further expected that in beverage compositions, a blend of monatin and erythritol further enhances citrus flavors and provides more favorable sweetness profiles, as compared to aspartame or sucralose. It is expected that blends of monatin and erythritol will exhibit these benefits in any beverage composition, such as soft drinks, carbonated beverages, syrups, dry beverage mixes, and slush beverages maintained at lower temperatures.

EXAMPLE 24: Evaluation of R, R Monatin in Beverages

Beverages (cola, lemon-lime and orange) were formulated and sweetened with aspartame, sucralose or R,R monatin. Qualitative evaluation was carried out.

Product Formulations

Soft drink formulations developed and evaluated are presented in Table 20. The term "throw" refers to dilution in water. For example, a throw of "1+4" means 1 part concentrate formulation to 4 parts water. Thus, if a concentrate formulation includes 0.021 % wt/vol (i.e., 210 ppm) of R,R monatin, for example, a throw of 1+4 makes a diluted beverage containing 42 ppm (210 ppm/5) R,R monatin.

Table 20. Soft drink formulations (concentrates)

Flavor	Ingredient	Concentration (%; w/v)
Lemon/Lime	L/L flavor: 76291-76	0.55
	Citric acid	0.80
	Sodium citrate	0.10
	Sodium benzoate (20% solution)	0.38
	Sweetener	(i) Aspartame 0.250 (ii) Sucralose 0.100 (iii) R,R Monatin 0.021
	Water	To volume
	Throw	1+4
Orangeade	Orange juice concentrate (6x)	5.420
	Citric acid	2.600
	Sodium citrate	0.520
	Orange flavor 2SX-73268	0.650
	β-carotene 0F0996	0.100
	Sodium benzoate (20% solution)	0.488
	Sweetener	(i) Aspartame 0.3575 (ii) Sucralose 0.1430 (iii) R,R Monatin 0.0293
	Water	To volume
	Throw	1+5.5
Cola	Cola flavor C40385	0.7150
	Cola flavor C40386	0.7150
	Sodium benzoate (20% solution)	0.3750
	Sweetener	(i) Aspartame 0.275 (ii) Sucralose 0.110 (iii) R,R Monatin 0.0225
	Water	To volume
	Throw	1+4

Final ready-to-drink beverages (after throw) contained sweetener concentrations as follows:

Lemon/lime	Aspartame	500 ppm
	Sucralose	200 ppm
	R,R Monatin	42 ppm
Orangeade	Aspartame	550 ppm
	Sucralose	220 ppm
	R,R Monatin	45 ppm
Cola	Aspartame	550 ppm
	Sucralose	220 ppm
	R,R Monatin	45 ppm

Sensory Evaluation of Beverages

Evaluation of these soft drinks was carried out by a panel (n = 6) of assessors who evaluated each set of drinks on separate tasting occasions. Results of the evaluations are summarized in Table 21.

Table 21. Sensory evaluation of soft drinks

Product	Sweetener/concentration	Comments
Lemon/lime	Aspartame/500ppm	Balanced sweetness/acidity profile. Very low level of bitterness. Pleasant fruity flavor. Typical APM aftertaste that is perceived at the back of the tongue.
	Sucralose/200ppm	Slow sweetness onset allows stronger lemon/lime notes to be perceived up front. Strong lingering sweet, cloying aftertaste that cuts through the flavor and leaves no pleasant fruity aftertaste.
	Monatin/42ppm	Balanced sweetness/acidity profile, but lower levels of perceived lemon/lime flavor up-front.
Orangeade	Aspartame/550ppm	Good temporal characteristics although the typical aspartame flavor is clearly apparent. No evidence of flavor enhancement.
	Sucralose/220ppm	Delay in sweetness onset means first impressions are of acidity. Product flavor and overall impression somewhat out of balance because of sweetness profile not matching acidity or flavor profiles.
	Monatin/45ppm	Good temporal characteristics although an aftertaste flavor typical of aspartame is apparent. No evidence of strong flavor enhancement. Overall, judged very similar qualitatively to aspartame.
Cola	Aspartame/550ppm	Good temporal characteristics although the typical aspartame flavor is clearly apparent. Good sweet/acid balance.
	Sucralose/220ppm	Delay in sweetness onset means first impressions are of acidity. Product flavor and overall impression somewhat out of balance because of sweetness profile did not match acidity or flavor profiles.
	Monatin/45ppm	Overall, judged quite similar qualitatively to aspartame. Onset of monatin seems slightly delayed, which makes the product slightly out of balance. No evidence of strong flavor enhancement.

Discussion

In lemon/lime, orangeade and cola beverages, monatin delivered a sweet taste similar in quality to aspartame and slightly better than that of sucralose, both of which are high quality sweeteners. In the lemon/lime beverage, less aftertaste was noted in the monatin formulation than in the aspartame formulation. Moreover, the potency of R,R monatin is greater than that of aspartame and sucralose.

EXAMPLE 25: Sweetness dose response curve of monatin and saccharin

Sweetness of monatin and saccharin was assessed using 20 trained sensory evaluators, making judgements in duplicate. Test and reference solutions were prepared in citric/citrate buffer at pH 3.2. *See* FIG. 16. The more linear response of R,R/S,S monatin, as compared to saccharin, is consistent with the delivery of a more sugar-like taste character. The plateau above 10% SEV indicates absence/low levels of "mixture-suppressing" off-tastes and aftertastes. The shape of monatin's dose-response curve is similar to those of aspartame, sucralose and alitame, all of which are "quality" sweeteners.

With R,R/S,S monatin as a sole sweetener in the model system (pH 3.2), the following characteristics were observed: (1) slight delay in sweet taste onset; (2) sweet taste decay was quite rapid; (3) slight "aspartame-like" aftertaste, slightly sweet aftertaste, no bitterness in the aftertaste; and (4) residual cooling sensation in un-flavored systems.

EXAMPLE 26: Stability of monatin at pH 3 with increasing temperatures

A sample of synthetic monatin was subjected to pH 3 at temperatures of 25°C, 50°C and 100°C. At room temperature and pH 3, a 14% loss in monatin was observed over a period of 48 hours. This loss was attributed to lactone formation. At 50°C and pH 3, a 23% loss in monatin was observed over a period of 48 hours. This loss was attributed to lactone formation and the buildup of an unknown compound after about 15.5 minutes. At 100°C and pH 3, nearly all monatin was lost after 24 hours. The major detectable component was an unknown at 15.5 minutes.

EXAMPLE 27: Sensory stability of monatin and aspartame at pH 2.5, 3.0, 4.0 at 40°C

The sensory stability of monatin solutions prepared at pH 2.5, 3.0 and 4.0 and stored at 40°C was monitored for 100 days. Loss of sweetness from these solutions was compared with the losses of sweetness from aspartame solutions prepared and stored under identical conditions.

The sensory stability of monatin (8% SEV, ~55 ppm, synthetic blend containing approximately 96% of the 2R,4R/2S, 4S enantiometric pair and 4% of the 2R,4S/2S,4R enantiometric pair) in phosphate/citrate buffers having a pH of 2.5, 3.0, and 4.0 was examined after storage at 40°C. The stability of monatin was compared to that of aspartame (400 ppm) in the same buffers. Three sucrose reference solutions were prepared in the same phosphate/citrate buffers as the monatin and aspartame solutions. All prepared solutions were stored in the dark.

Buffer compositions: pH 2.5 Phosphoric acid (75% solution) 0.127% (w/v)
Tri-sodium citrate monohydrate 0.005% (w/v)
pH 3.0 Phosphoric acid (75% solution) 0.092% (w/v)
Tri-sodium citrate monohydrate 0.031% (w/v)
pH 4.0 Phosphoric acid (75% solution) 0.071% (w/v)
Tri-sodium citrate monohydrate 0.047% (w/v)

The sweetness of each sweetener relative to sucrose was assessed in duplicate by a panel (n = 8) of trained sensory evaluators experienced in the sweetness estimation procedure. All samples (in the same buffers) were served in duplicate at a temperature of 22°C ± 1°C. Monatin (test) solutions, coded with 3 digit random number codes were presented individually to panelists, in random order. Sucrose reference standards, ranging from 4.0 – 10.0% (w/v) sucrose, increasing in steps of 0.5% (w/v) sucrose were also provided. Panelists were asked to estimate sweetness by comparing the sweetness of the test solution to the sucrose standards. This was carried out by taking 3 sips of the test solution, followed by a sip of water, followed by 3 sips of sucrose standard followed by a sip of water, etc. Panelists were

encouraged to estimate the sweetness to one decimal place, e.g., 6.8, 8.5. A five minute rest period was imposed between evaluating the test solutions. Panelists were also asked to rinse well and eat a cracker to reduce any potential carry over effects.

Tables 22 and 23 present results of the stability studies in the phosphate citrate buffers. At each pH and after 100 days' storage at 40°C in the dark, the percentage retention of monatin sweetness was greater than that retained with aspartame. At pH 4.0, the loss of sweetness of the monatin solution appeared almost to have stabilized since there was very little change in measured sweetness intensity between Days 17 and 100, whereas the aspartame solution continued to lose sweetness.

TABLE 22

Sensory Stability of Monatin: Sweetness after 100 Days Storage at 40°C

A.

pH	Time (days)	SEV Monatin (% sucrose)	Retention of Monatin Sweetness (%)	SEV Aspartame (% sucrose)	Retention of Aspartame Sweetness (%)
2.5	0	7.35		7.34	
	1	6.86	93.3	6.90	94.0
	2	6.70	91.2	6.80	92.6
	3	6.50	88.4	6.60	89.9
	4	6.26	85.2	6.29	85.7
	7	6.08	82.7	6.01	81.9
	8	5.98	81.4	5.98	81.5
	9	5.89	80.1	5.97	81.3
	11	5.78	78.6	5.86	79.8
	50	4.61	62.7	4.19	57.1
	100	2.10	28.6	0.80	10.9

B.

pH	Time (days)	SEV Monatin (% sucrose)	Retention of Monatin Sweetness (%)	SEV Aspartame (% sucrose)	Retention of Aspartame Sweetness (%)
3.0	0	7.08		7.15	
	1	7.05	99.6	6.90	96.5
	2	6.60	93.2	6.87	96.1
	3	6.47	91.4	6.60	92.3
	4	6.49	91.6	6.43	89.9
	7	6.04	85.3	6.17	86.3
	8	5.93	83.8	5.93	82.9
	9	5.88	83.1	5.94	83.1
	11	5.88	83.1	5.83	81.5
	50	5.12	72.3	4.71	65.9
	100	4.10	57.9	2.20	30.8

C.

pH	Time (days)	SEV Monatin (% sucrose)	Retention of Monatin Sweetness (%)	SEV Aspartame (% sucrose)	Retention of Aspartame Sweetness (%)
4.0	0	7.40		7.10	
	3	7.08	95.7	6.75	95.1
	8	6.42	86.8	6.23	87.8
	11	6.36	85.9	6.02	84.8
	17	6.10	82.4	5.75	81.0
	24	6.25	84.5	5.85	82.4
	50	6.14	82.9	5.29	74.5
	100	5.80	78.4	4.10	57.7

TABLE 23

Stability: Amount of sweetness remaining after 100 days storage at stated pH at 40°C

pH	**Sweetener**	**Sweetness Retained (%)**
2.5	**Aspartame**	**11**
2.5	**Monatin**	**29**
3.0	**Aspartame**	**31**
3.0	**Monatin**	**58**
4.0	**Aspartame**	**58**
4.0	**Monatin**	**78**

The respective buffers were effective at maintaining pH, as seen in Table 24:

TABLE 24

Sweetener	Nominal pH	Actual pH (after 50 days)
Monatin	2.5	2.39
	3.0	3.13
	4.0	4.28
Aspartame	2.5	2.49
	3.0	3.13
	4.0	4.19

If a *pseudo*-first order breakdown reaction is assumed, a plot of $\log_n$ percentage retention versus time ($\log_n$%RTN v. t) allows estimation of the half-life ($t^1/_2$) and rate constant (k) of sweetness loss under any given set of conditions. In so doing, the kinetics of monatin and aspartame sweetness loss may be summarized as follows in Table 25.

TABLE 25

Sweetener	PH	Half-life ($t^1/_2$; days)	Rate constant (k; day^{-1})
Monatin	2.5	65 days	0.011day^{-1}
	3.0	115 days	0.006day^{-1}
	4.0	230 days	0.003day^{-1}
Aspartame	2.5	55 days	0.013day^{-1}
	3.0	75 days	0.009day^{-1}
	4.0	140 days	0.005day^{-1}

At each pH and after 100 days storage at 40°C, the percentage retention of monatin sweetness is greater than that retained from aspartame. At pH 4.0, the loss of sweetness of the monatin solution appears almost to have stabilized since there has been very little change in measured sweetness intensity between Days 17 and 100, whereas the aspartame solution continues to lose sweetness.

Estimates of the half-life of monatin and aspartame indicate that sweetness derived from monatin is lost at a slower rate than that from aspartame. Half-life estimates for monatin sweetness at pH 2.5, 3.0 and 4.0 were 65 days, 115 days and 230 days, respectively. Aspartame half-life estimates were 55 days, 75 days and 140 days under the same conditions.

Thus, under acidic conditions and storage at 40°C, monatin delivers a more stable sweetness than does aspartame. Monatin has a better stability than aspartame in colas and other beverages having a lower pH, as well as at higher temperatures. Because monatin exhibits better stability than aspartame, and reaches an equilibrium and does not irreversibly break down at pH 3, it is expected that monatin provides a long-term stable sweetness at a low pH in beverages, such as cola beverages.

It was further found (data not shown) that when exposed to ultra violet (UV) light, monatin in phosphoric/citrate buffer at pH 3.0 (at ambient temperature) is similarly stable or slightly more stable than aspartame. UV instability can be accelerated by certain flavor systems. UV-absorbing packaging material, colorants and/or antioxidants can protect against UV light-induced flavor interactions in monatin-containing beverages.

EXAMPLE 28: Chromatography of stereoisomers of monatin

Sample Preparation – Approximately 50-75 μg of lyophilized material was placed in a microcentrifuge tube. To this 1.0 mL of HPLC grade methanol was added. The solution was vortexed for 30 minutes, centrifuged and an aliquot of the supernatant was removed for analysis.

Reversed Phase HPLC – Chromatography of two distinct diastereomer peaks (R,R/S,S and R,S/S,R) was accomplished using a 2.1 x 250mm Xterra™ MS C_8 5μm (Waters Corporation) HPLC column. Detection was carried out using an Ultima™ triple quadrupole mass spectrometer from Micromass. Mobile phase was delivered by the following gradient:

Time (min)		0	9	16	20	21
0.05%TFA	A%	95	65	10	10	95
Methanol, 0.05% TFA	B%	5	35	90	90	5
Flow mL/min		0.25	0.25	0.25	0.25	0.25

Chiral HPLC – Chromatography of two distinct monatin stereoisomers (R,R and S,S) was accomplished using a 250 x 4.6 mm Chirobiotic T(Advanced Separations Technologies, Inc.) HPLC column. Detection was carried out using an Ultima™ triple quadrupole mass spectrometer from Micromass. Mobile phase consisted of Methanol with 0.2% Acetic acid and 0.05% ammonium hydroxide.

Mass Spectrometry (MS/MS) – The presence of monatin was detected by a Selected Reaction Monitoring (SRM) experiment. The protonated molecular ion of monatin ($[M+H]^+$) has a *m/z*=293.3. Fragmentation of this molecular ion produces a significant ion at *m/z*=257.3 arising from multiple dehydrations of the molecular ion. This transition has been shown to be very specific to monatin and was chosen as the transition (293.3 to 257.3) for monitoring during the SRM experiment. This method of detection was employed for both reversed phase and chiral separations of monatin.

Results – The standard samples of R,S/S,R and S,S/R,R were evaluated under Reversed Phase HPLC. The samples were prepared by derivatization and enzymatic resolution. Chromatograms for standard solutions are displayed in FIG. 17. Following the reversed phase analysis, chiral chromatography was performed to evaluate specific stereoisomers present in the samples. Chiral chromatography of standard S,S and R,R, monatin solutions are displayed in FIG. 18.

EXAMPLE 29: Stability of monatin at high temperature (80 °C) and neutral pH

A 100 milliliter solution of 75 ppm monatin at pH 7 was used as a stock solution. The synthetic monatin sample contained approximately 96% of the 2R,4R/2S,4S enantiomeric pair and 4% of the 2R,4S/2S,4R enantiomeric pair. Samples were incubated at 80°C and pH 7 for the duration of the experiment and samples were withdrawn at 0, 1, 2, 3, 4 hours and 1, 2, 4, 7, 14, 21 and 35 days. All experimental conditions were run in duplicate.

Separation and Quantification Using LC-MS using Reverse Phase Chromatography – A response curve was established for both detected diastereomer peaks of the synthetic monatin. A range of 5-150 ppm was bracketed with the synthetic monatin standard dissolved in DI water. Separation of the two diastereomer peaks was accomplished using a 3.9 x 150mm Novapak C18 (Waters Corporation) HPLC column. Ultraviolet-Visible (UV) and Mass Spectrometer (MS) detectors were used in series for detection and quantitation. Monatin and its lactone peak each have a UV_{max} at 279 nm that aided in precise detection. Quantification was done by acquiring Selected Ion Monitoring (SIM) scan of 293.3 m/z and 275.3 m/z in positive-ion electrospray mode.

Results – At a neutral pH, the degree of degradation of monatin was determined to be insignificant even after 7-35 days. The disappearance of monatin over time is highly dependent on pH since the primary byproducts are cyclization and possibly very small levels of racemization. During the experiment at 80°C and pH 7, no change in concentration of racemic RR/SS monatin or lactones thereof was detected within the limits of precision afforded by using LC-MS for quantitation.

Due to the thermal stability of monatin at neutral pH, it is expected that monatin has a suitable stability for beverages at a neutral pH (such as dairy or powdered beverage compositions). It is also expected that monatin has longer shelf life in these beverage compositions, as compared to other high intensity sweeteners (e.g., aspartame). In addition, it is expected that monatin will be more stable during processing conditions, such as heat filling.

EXAMPLE 30: Other Soft Drink Formulations (Concentrates)

Formulation A:

Ingredient	Concentration (%; wt/vol)
Cola flavor C40385	0.7150
Cola flavor C40386	0.7150
Sodium benzoate (20% solution)	0.3750
S,S monatin	0.99
Water	To volume

Throw 1+4. The diluted ready-to-drink beverage contains 1980 ppm S,S monatin.

Formulation B:

Ingredient	Concentration (%; wt/vol)
Cola flavor C40385	0.7150
Cola flavor C40386	0.7150
Sodium benzoate (20% solution)	0.3750
Monatin (racemic mix)	0.04
Water	To volume

Throw 1+4. The diluted ready-to-drink beverage contains 80 ppm of monatin racemic mix.

Formulation C:

Ingredient	Concentration (%; wt/vol)
Cola flavor C40385	0.7150
Cola flavor C40386	0.7150
Sodium benzoate (20% solution)	0.3750
S,S monatin	0.275
R,R monatin	0.016
Water	To volume

Throw 1+4. The diluted ready-to-drink beverage contains 550 ppm S,S monatin and 32 ppm R,R monatin.

In view of the many possible embodiments to which the principles of this disclosure may be applied, it should be recognized that the illustrated embodiments are only particular examples of the disclosure and should not be taken as a limitation on the scope of the disclosure.

Claims:

1. A beverage composition comprising monatin or salt thereof.

2. The beverage composition of claim 1, wherein an amount of the composition contains less calories and carbohydrates than the same amount of the beverage composition containing sucrose or high fructose corn syrup in place of the monatin or salt thereof at comparable sweetness.

3. The beverage composition of claim 1, wherein the composition further comprises a citrus flavor, and wherein the monatin or salt thereof is present in an amount that enhances the flavor provided by the citrus flavor.

4. The beverage composition of claim 1, wherein the composition further comprises a citrus flavor and a carbohydrate, and wherein the monatin or salt thereof and the carbohydrate are present in an amount that enhances the flavor provided by the citrus flavor.

5. The beverage composition of claim 4, wherein the carbohydrate is chosen from erythritol, maltodextrin, sucrose and a combination thereof.

6. A carbonated beverage, comprising a syrup composition in an amount ranging from about 15% to about 25% by weight of the carbonated beverage, wherein the syrup composition comprises monatin or salt thereof.

7. The beverage composition of claim 1, wherein the composition comprises from about 3 to about 10000 ppm monatin or salt thereof.

8. The beverage composition of claim 1, wherein the beverage composition is a syrup or dry beverage mix, and wherein the composition comprises from about 10 to about 10000 ppm monatin or salt thereof.

9. The beverage composition of claim 8, wherein the beverage composition is a syrup, and wherein the syrup is a concentrate adapted for dilution in a drink in a range of about 1 part syrup:3 parts drink to about 1 part syrup:5.5 drink.

10. The beverage composition of claim 9, wherein the syrup comprises from about 600 to about 10000 ppm S,S monatin or salt thereof.

11. The beverage composition of claim 9, wherein the syrup comprises from about 18 to about 300 ppm R,R monatin or salt thereof.

12. The beverage composition of claim 1, wherein the beverage composition is a syrup comprising from about 0 to about 10000 ppm S,S monatin or salt thereof, and from 0 to about 300 ppm R,R monatin or salt thereof.

13. The beverage composition of claim 1, wherein the beverage composition is a dry beverage mix comprising from about 10 to about 10000 ppm monatin or salt thereof.

14. The beverage composition of claim 13, wherein the dry beverage mix comprises from about 600 to about 10000 ppm S,S monatin or salt thereof.

15. The beverage composition of claim 13, wherein the dry beverage mix comprises from about 10 to about 450 ppm R,R monatin or salt thereof.

16. The beverage composition of claim 1, wherein the beverage composition is a dry beverage mix comprising from about 0 to about 10000 ppm S,S monatin or salt thereof, and from about 0 to about 450 ppm R,R monatin or salt thereof.

17. The beverage composition of claim 7, wherein the composition is substantially free of R,R monatin or salt thereof.

18. The beverage composition of claim 7, wherein the composition is substantially free of S,S monatin or salt thereof.

19. The beverage composition of claim 1, wherein the composition comprises from about 3 to about 450 ppm R,R monatin or salt thereof.

20. The beverage composition of claim 19, wherein the composition comprises from about 6 to about 225 ppm R,R monatin or salt thereof.

21. The beverage composition of claim 1, wherein the composition comprises from about 3 to about 10000 ppm S,S monatin or salt thereof.

22. The beverage composition of claim 21, wherein the composition comprises from about 60 to about 4600 ppm of S,S monatin or salt thereof.

23. The beverage composition of claim 1, wherein the composition comprises from about 0 to about 10000 ppm of S,S monatin or salt thereof, and from about 0 to about 450 ppm R,R monatin or salt thereof.

24. The beverage composition of claim 1, where the composition is a ready-to-drink composition comprising from about 3 to about 2000 ppm monatin or salt thereof.

25. The ready-to-drink composition of claim 24, where the ready-to-drink composition comprises from about 5 to about 50 ppm R,R monatin or salt thereof.

26. The ready-to-drink composition of claim 24 , where the ready-to-drink composition comprises from about 60 to about 2000 ppm S,S monatin or salt thereof.

27. The beverage composition of claim 1, wherein the composition comprises about 450 or less ppm R,R monatin or salt thereof, and wherein the monatin or salt thereof is substantially free of S,S, S,R or R,S monatin or salt thereof.

28. The beverage composition of claim 1, wherein the composition comprises about 10000 or less ppm S,S monatin or salt thereof, and wherein the monatin or salt thereof is substantially free of R,R, S,R or R,S monatin or salt thereof.

29. The beverage composition of claim 1, wherein the monatin or salt thereof consists essentially of R,R monatin or salt thereof.

30. The beverage composition of claim 1, wherein the monatin or salt thereof consists essentially of S,S monatin or salt thereof.

31. The beverage composition of claim 1, wherein the monatin or salt thereof is a stereoisomerically-enriched R,R monatin or salt thereof.

32. The beverage composition of claim 1, wherein the monatin or salt thereof is a stereoisomerically-enriched S,S monatin or salt thereof.

33. The beverage composition of claim 1, wherein the monatin or salt thereof comprises at least 95% R,R monatin or salt thereof.

34. The beverage composition of claim 1, wherein the monatin or salt thereof comprises at least 95% S,S monatin or salt thereof.

35. The beverage composition of claim 1, wherein the monatin or salt thereof is produced in a biosynthetic pathway.

36. The beverage composition of claim 1, wherein the beverage composition further comprises erythritol, trehalose, a cyclamate, D-tagatose or combination thereof.

37. The beverage composition of claim 1, wherein the composition is non-cariogenic.

38. A beverage composition comprising a stereoisomerically-enriched monatin mixture, wherein the monatin mixture is produced via a biosynthetic pathway.

39. The beverage composition of claim 38, wherein the biosynthetic pathway is a multi-step pathway and at least one step of the multi-step pathway is a chemical conversion.

40. The beverage composition of claim 38, wherein the mixture is predominantly R,R monatin or salt thereof.

41. The beverage composition of claim 38, wherein the mixture is predominantly S,S monatin or salt thereof.

42. A beverage composition comprising a monatin composition produced in a biosynthetic pathway, wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants.

43. A beverage composition comprising monatin or salt thereof, wherein the monatin or salt thereof is produced in a biosynthetic pathway and isolated from a recombinant cell, and wherein the recombinant cell does not contain petrochemical, toxic or hazardous contaminants.

44. The beverage composition of claim 1, wherein the monatin or salt thereof is a blend of R,R and S,S, monatin or salt thereof.

45. The beverage composition of claim 1, wherein the composition further comprises a bulk sweetener, a high-intensity sweetener, a lower glycemic carbohydrate, a flavoring, an antioxidant, caffeine, a sweetness enhancer or a combination thereof.

46. The beverage composition of claim 45,

wherein the flavoring is chosen from a cola flavor, a citrus flavor and a combination thereof,

wherein the bulk sweetener is chosen from corn sweeteners, sucrose, dextrose, invert sugar, maltose, dextrin, maltodextrin, fructose, levulose, high fructose corn syrup, corn syrup solids, levulose, galactose, trehalose, isomaltulose, fructo-oligosaccharides and a combination thereof,

wherein the high-intensity sweetener is chosen from sucralose, aspartame, saccharin, acesulfame K, alitame, thaumatin, dihydrochalcones, neotame, cyclamates, stevioside, mogroside, glycyrrhizin, phyllodulcin, monellin, mabinlin, brazzein, circulin, pentadin and a combination thereof,

wherein the lower glycemic carbohydrate is chosen from D-tagatose, sorbitol, mannitol, xylitol, lactitol, erythritol, maltitol, hydrogenated starch hydrolysates, isomalt, D-psicose, 1,5 anhydro D-fructose and a combination thereof, and

wherein the sweetness enhancer is chosen from curculin, miraculin, cynarin, chlorogenic acid, caffeic acid, strogins, arabinogalactan, maltol, dihyroxybenzoic acids and a combination thereof.

47. The beverage composition of claim 1, wherein the beverage composition comprises a blend of monatin or salt thereof and a non-monatin sweetener.

48. The beverage composition of claim 47, wherein the non-monatin sweetener is chosen from sucrose and high fructose corn syrup.

49. A method for making a beverage composition comprising monatin or salt thereof, wherein the method comprises producing monatin or salt thereof from at least one substrate chosen from glucose, tryptophan, indole-3-lactic acid, indole-3-pyruvate and the monatin precursor.

50. A method for making a beverage composition comprising monatin or salt thereof, wherein the method comprises producing monatin or salt thereof through a biosynthetic pathway.

51. A method for making a beverage composition comprising monatin or salt thereof, wherein the method comprises producing monatin or salt thereof using at least one biological conversion.

52. A method for making a beverage composition comprising monatin or salt thereof, wherein the method comprises producing monatin or salt thereof using only biological conversions.

53. The method of claim 49, wherein the method further comprises combining the monatin or salt thereof with at least one other ingredient that is not monatin or salt thereof.

54. The method of claim 49, wherein the method further comprises combining the monatin or salt thereof with erythritol, trehalose, a cyclamate, D-tagatose, maltodextrin or combination thereof.

55. The method of claim 53, wherein the at least one other ingredient is chosen from bulking agents, bulk sweeteners, liquid sweeteners, lower glycemic carbohydrates, high intensity sweeteners, thickeners, fats, oils, emulsifiers, antioxidants, sweetness enhancers, colorants, flavorings, caffeine, acids, powders, flow agents, buffers, protein sources, flavor enhancers, flavor stabilizers and a combination thereof.

56. The method of claim 53, wherein the beverage composition comprises from about 0 to about 10000 ppm of S,S monatin or salt thereof, and from about 0 to about 450 ppm R,R monatin or salt thereof.

57. A method for making a beverage composition comprising a monatin composition, wherein the method comprises: (a) producing monatin or salt thereof in a biosynthetic pathway in a recombinant cell; (b) isolating the monatin composition from the recombinant cell, wherein the monatin composition consists of monatin or salt thereof and other edible or potable material.

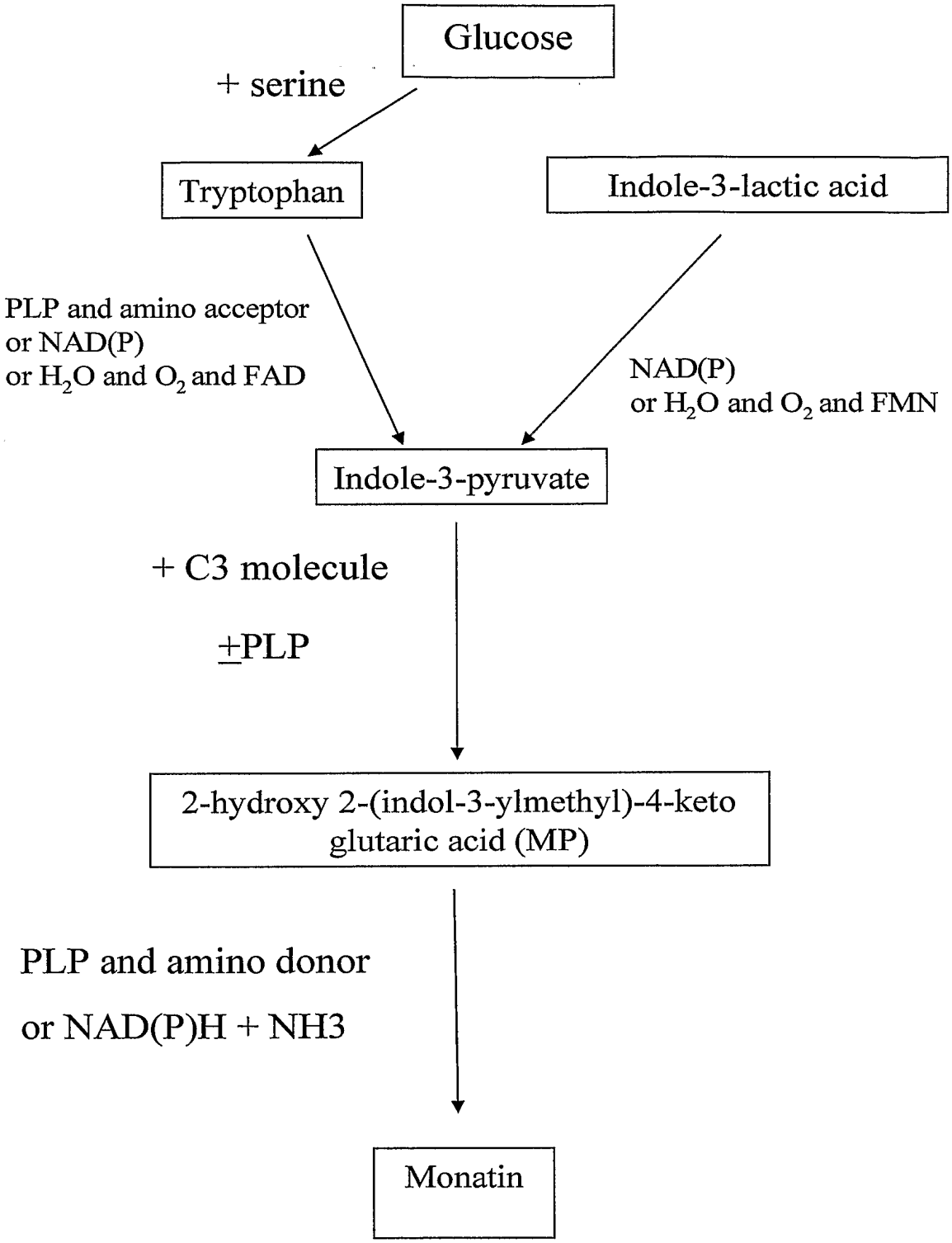

Glucose
+ serine
Tryptophan
Indole-3-lactic acid
PLP and amino acceptor
or NAD(P)
or H_2O and O_2 and FAD
NAD(P)
or H_2O and O_2 and FMN
Indole-3-pyruvate
+ C3 molecule
±PLP
2-hydroxy 2-(indol-3-ylmethyl)-4-keto
glutaric acid (MP)
PLP and amino donor
or NAD(P)H + NH3
Monatin

FIG. 1

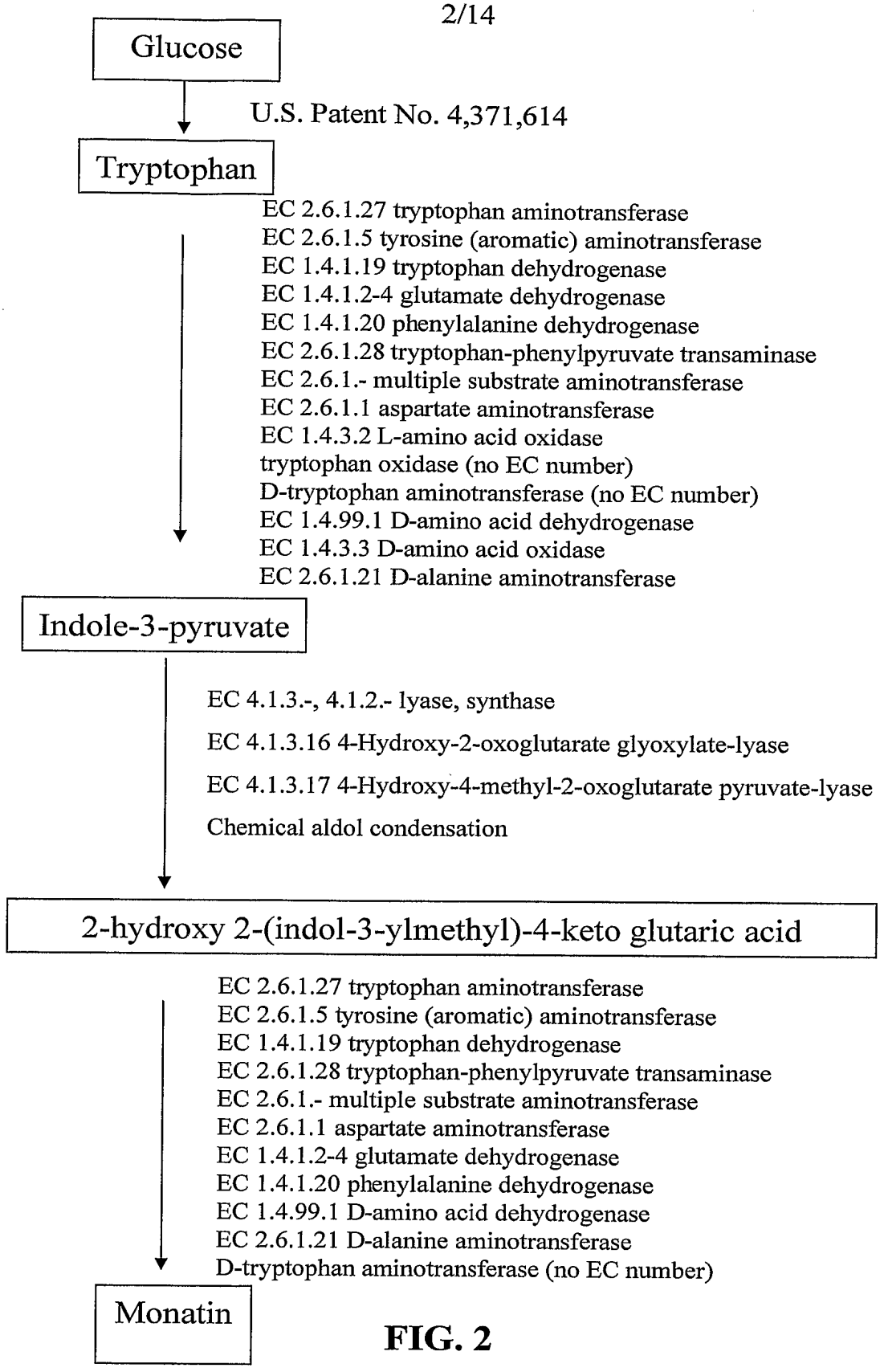

Glucose
U.S. Patent No. 4,371,614
Tryptophan
EC 2.6.1.27 tryptophan aminotransferase
EC 2.6.1.5 tyrosine (aromatic) aminotransferase
EC 1.4.1.19 tryptophan dehydrogenase
EC 1.4.1.2-4 glutamate dehydrogenase
EC 1.4.1.20 phenylalanine dehydrogenase
EC 2.6.1.28 tryptophan-phenylpyruvate transaminase
EC 2.6.1.- multiple substrate aminotransferase
EC 2.6.1.1 aspartate aminotransferase
EC 1.4.3.2 L-amino acid oxidase
tryptophan oxidase (no EC number)
D-tryptophan aminotransferase (no EC number)
EC 1.4.99.1 D-amino acid dehydrogenase
EC 1.4.3.3 D-amino acid oxidase
EC 2.6.1.21 D-alanine aminotransferase
Indole-3-pyruvate
EC 4.1.3.-, 4.1.2.- lyase, synthase
EC 4.1.3.16 4-Hydroxy-2-oxoglutarate glyoxylate-lyase
EC 4.1.3.17 4-Hydroxy-4-methyl-2-oxoglutarate pyruvate-lyase
Chemical aldol condensation
2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid
EC 2.6.1.27 tryptophan aminotransferase
EC 2.6.1.5 tyrosine (aromatic) aminotransferase
EC 1.4.1.19 tryptophan dehydrogenase
EC 2.6.1.28 tryptophan-phenylpyruvate transaminase
EC 2.6.1.- multiple substrate aminotransferase
EC 2.6.1.1 aspartate aminotransferase
EC 1.4.1.2-4 glutamate dehydrogenase
EC 1.4.1.20 phenylalanine dehydrogenase
EC 1.4.99.1 D-amino acid dehydrogenase
EC 2.6.1.21 D-alanine aminotransferase
D-tryptophan aminotransferase (no EC number)
Monatin

FIG. 2

FIG. 3

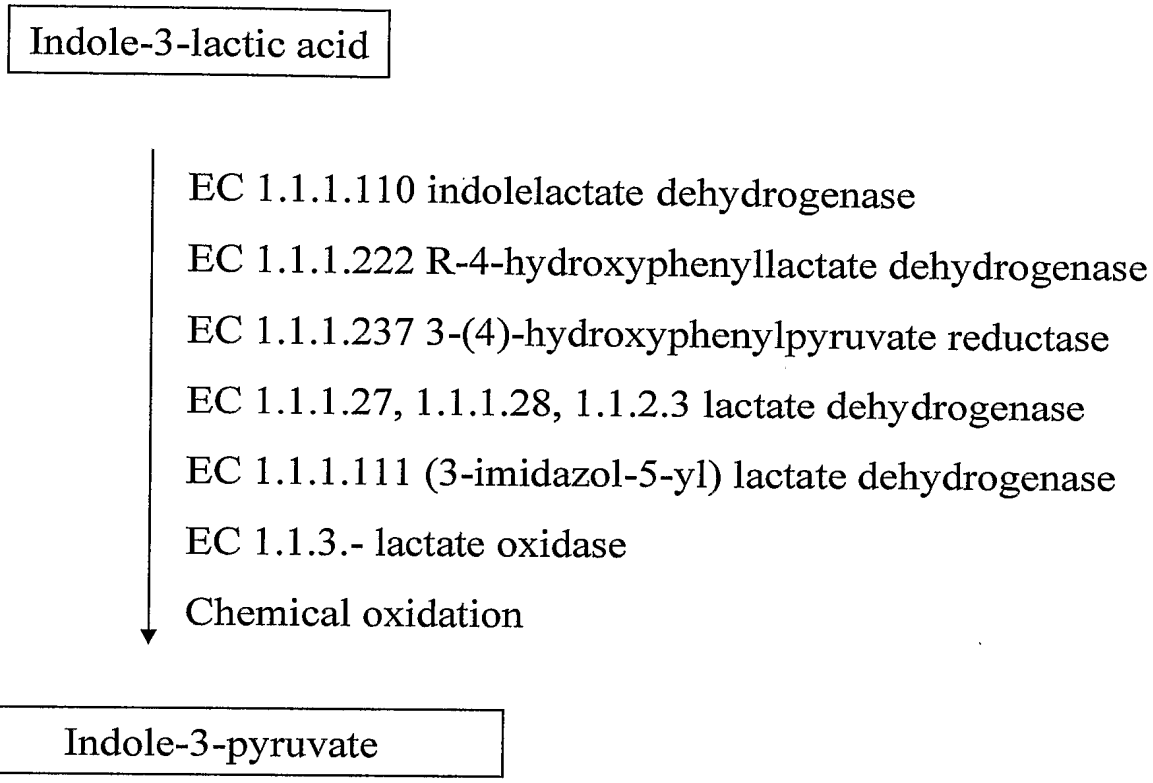
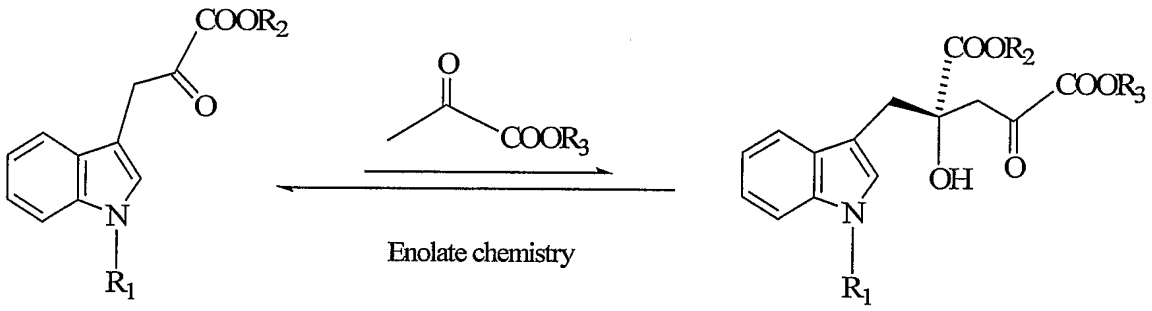


FIG. 4



R_1 = Boc, Cbz, etc.
R_2 and R_3 = Alkyl, Aryl, etc.

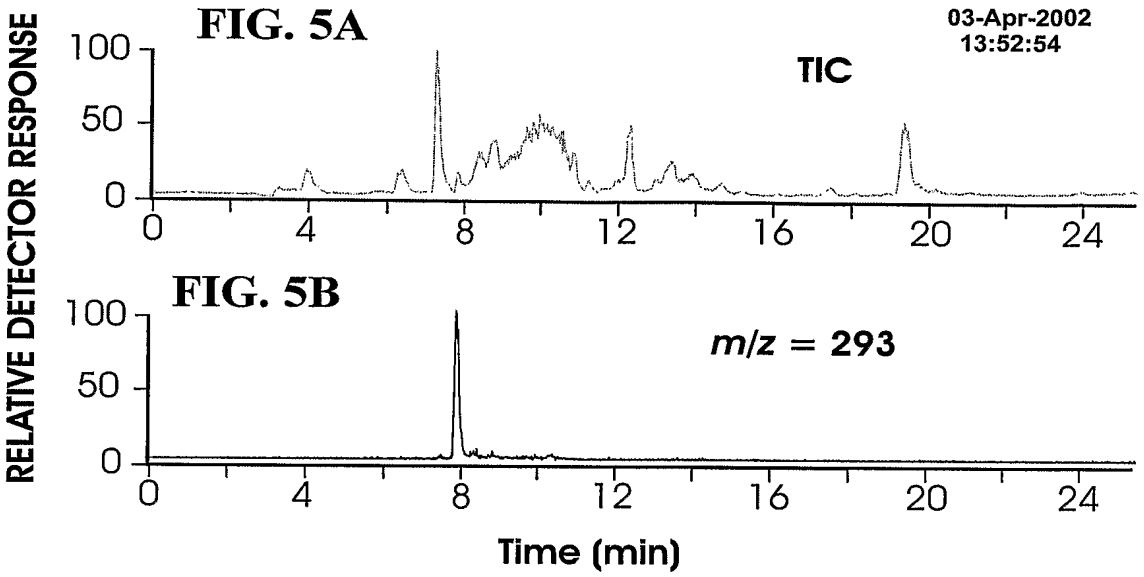


FIG. 5A
03-Apr-2002
13:52:54
TIC
100
50
0
0
4
8
12
16
20
24
FIG. 5B
m/z = 293
RELATIVE DETECTOR RESPONSE
Time (min)

FIG. 6

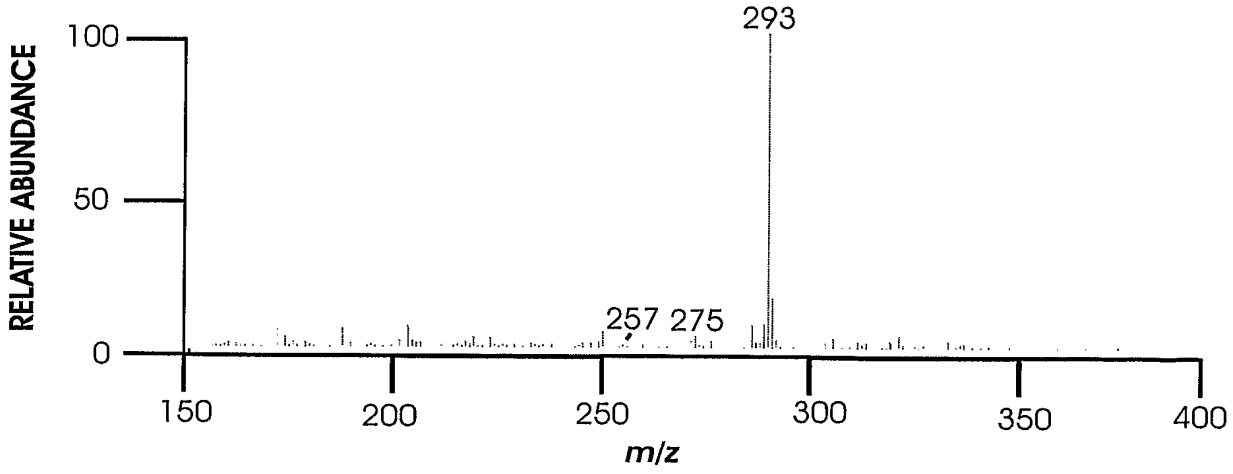

293
100
50
0
257 275
150
200
250
300
350
400
RELATIVE ABUNDANCE
m/z

FIG. 7A

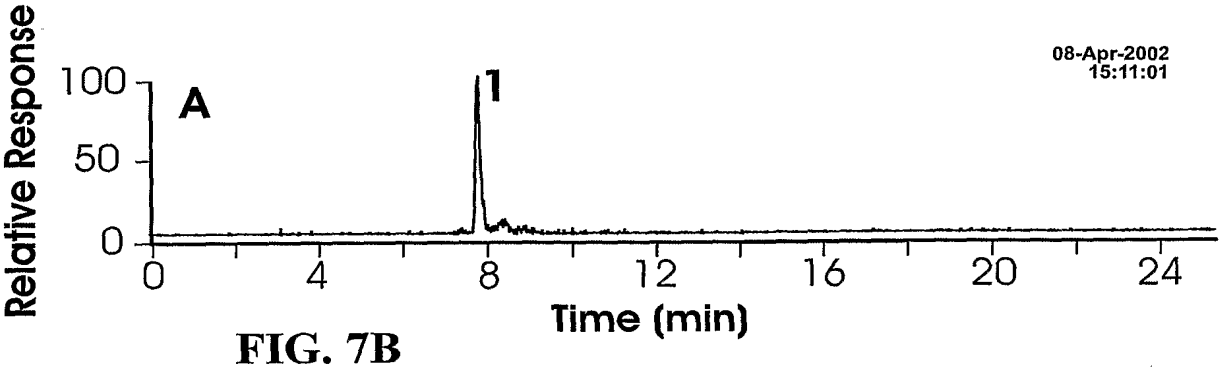


FIG. 7B

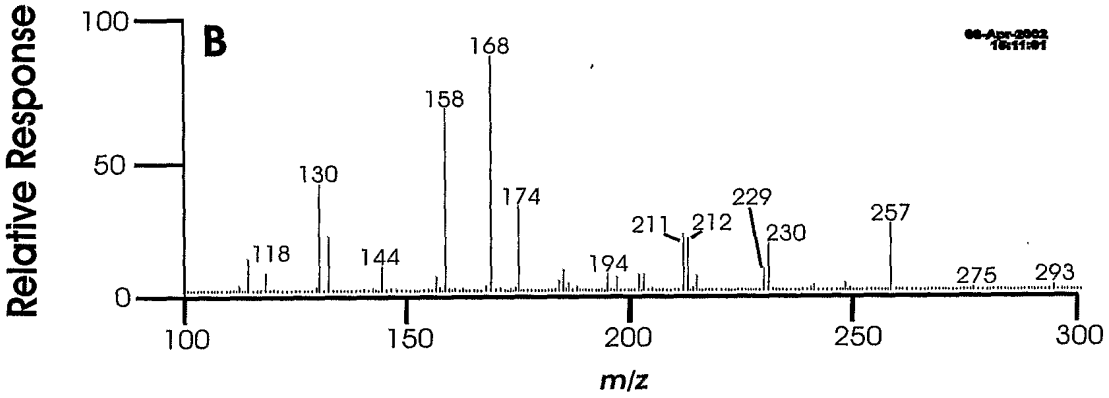
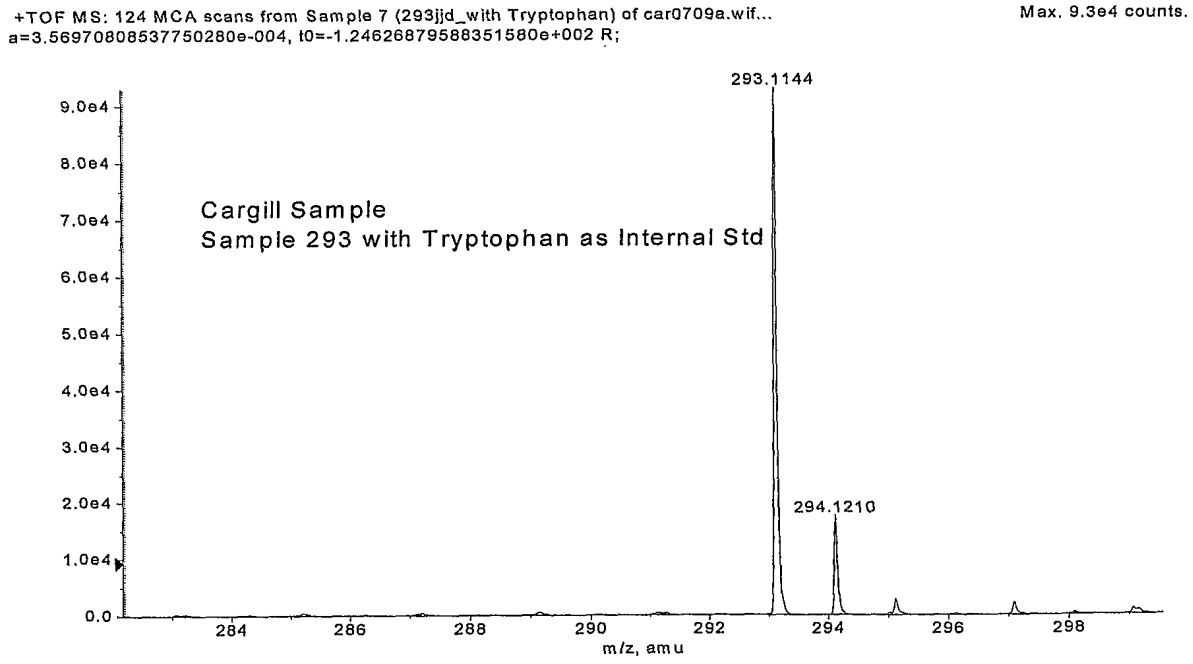


FIG. 8



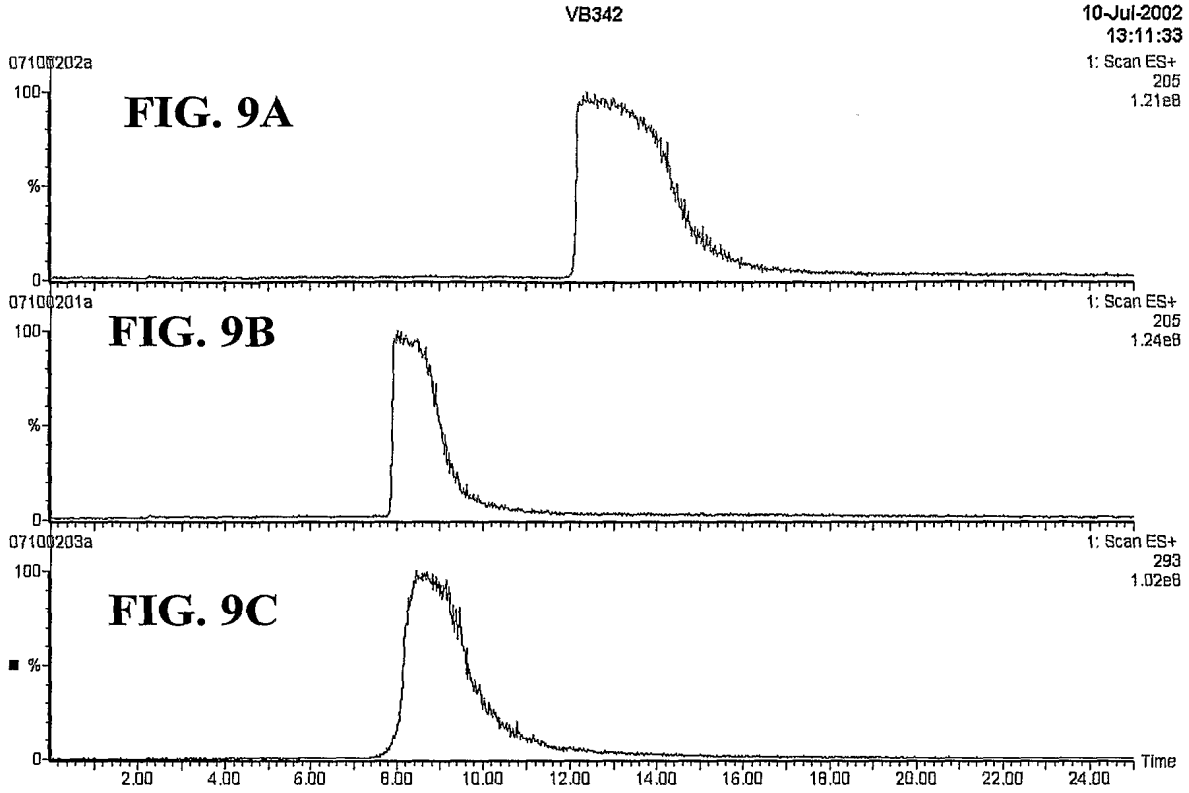

VB342
10-Jul-2002
13:11:33
071002202a
1: Scan ES+
205
1.21e8
100
%
0
FIG. 9A
071002201a
1: Scan ES+
205
1.24e8
FIG. 9B
071002203a
1: Scan ES+
293
1.02e8
FIG. 9C
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
Time

FIG. 10

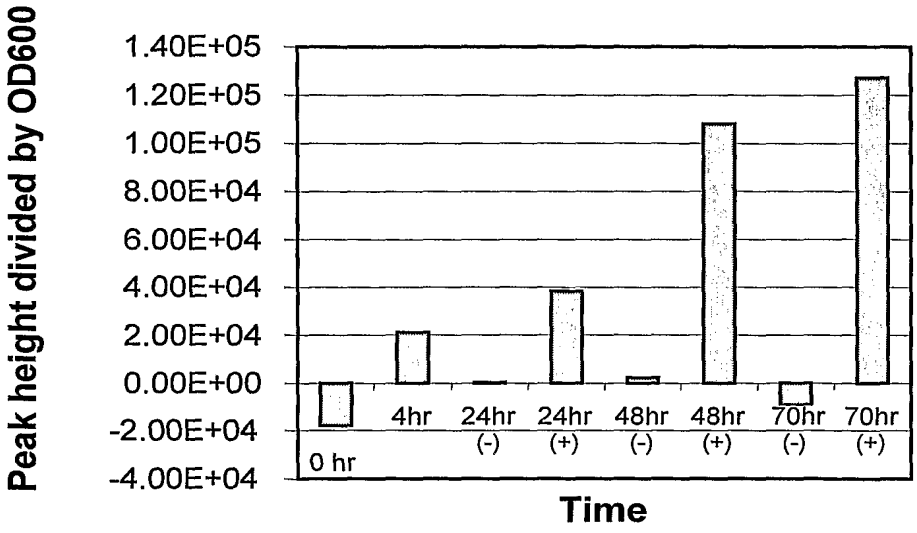

Peak height divided by OD600
1.40E+05
1.20E+05
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
-2.00E+04
-4.00E+04
0 hr
4hr
24hr (-)
24hr (+)
48hr (-)
48hr (+)
70hr (-)
70hr (+)
Time

FIG. 11

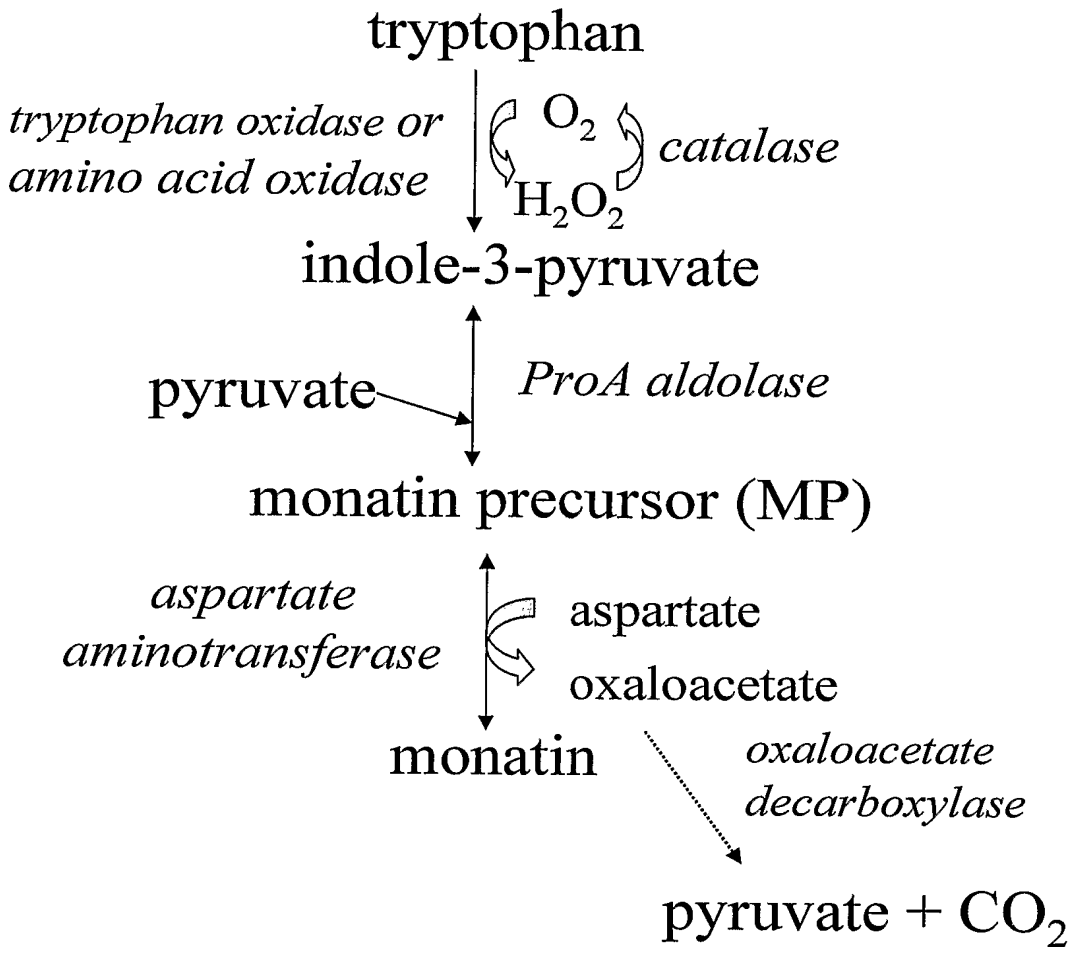

tryptophan
tryptophan oxidase or
amino acid oxidase
O2
H2O2
catalase
indole-3-pyruvate
pyruvate
ProA aldolase
monatin precursor (MP)
aspartate
aminotransferase
aspartate
oxaloacetate
monatin
oxaloacetate
decarboxylase
pyruvate + CO2

FIG. 12

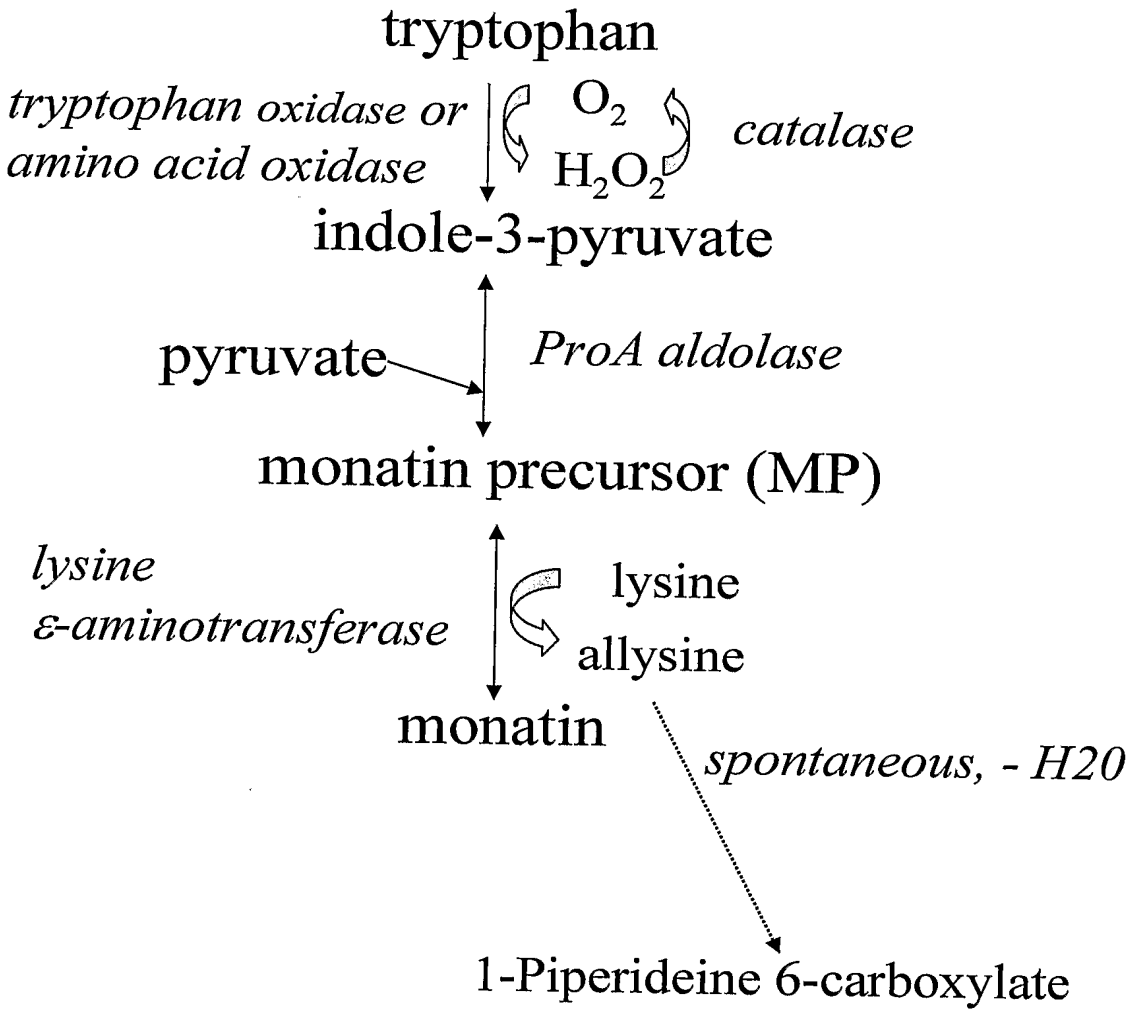

tryptophan
tryptophan oxidase or
amino acid oxidase
O2
H2O2
catalase
indole-3-pyruvate
pyruvate
ProA aldolase
monatin precursor (MP)
lysine
ε-aminotransferase
lysine
allysine
monatin
spontaneous, - H20
1-Piperideine 6-carboxylate

FIG. 13

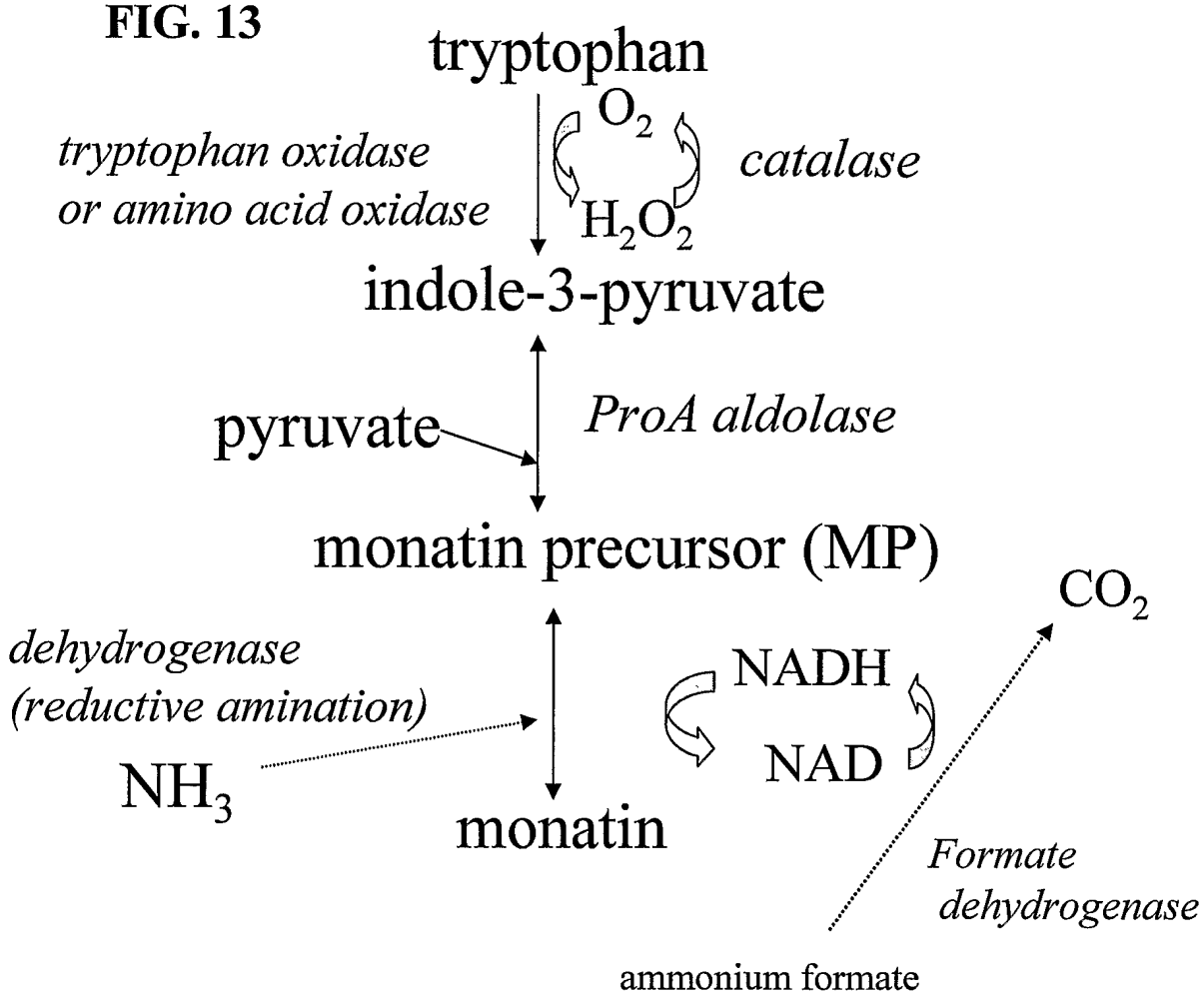

tryptophan
O2
tryptophan oxidase
or amino acid oxidase
catalase
H2O2
indole-3-pyruvate
pyruvate
ProA aldolase
monatin precursor (MP)
CO2
dehydrogenase
(reductive amination)
NADH
NAD
NH3
monatin
Formate
dehydrogenase
ammonium formate

FIG. 14:

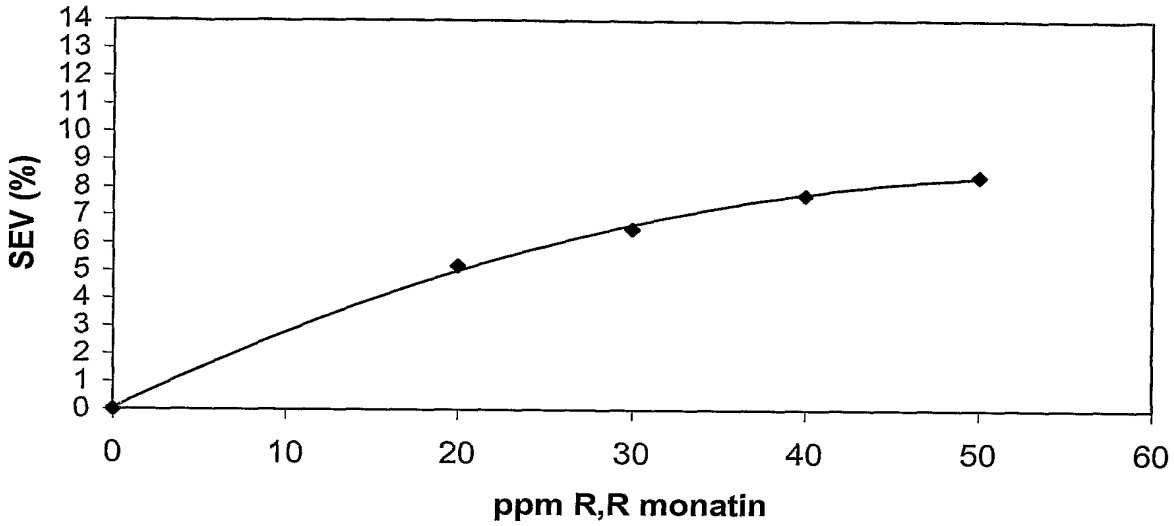


FIG. 15

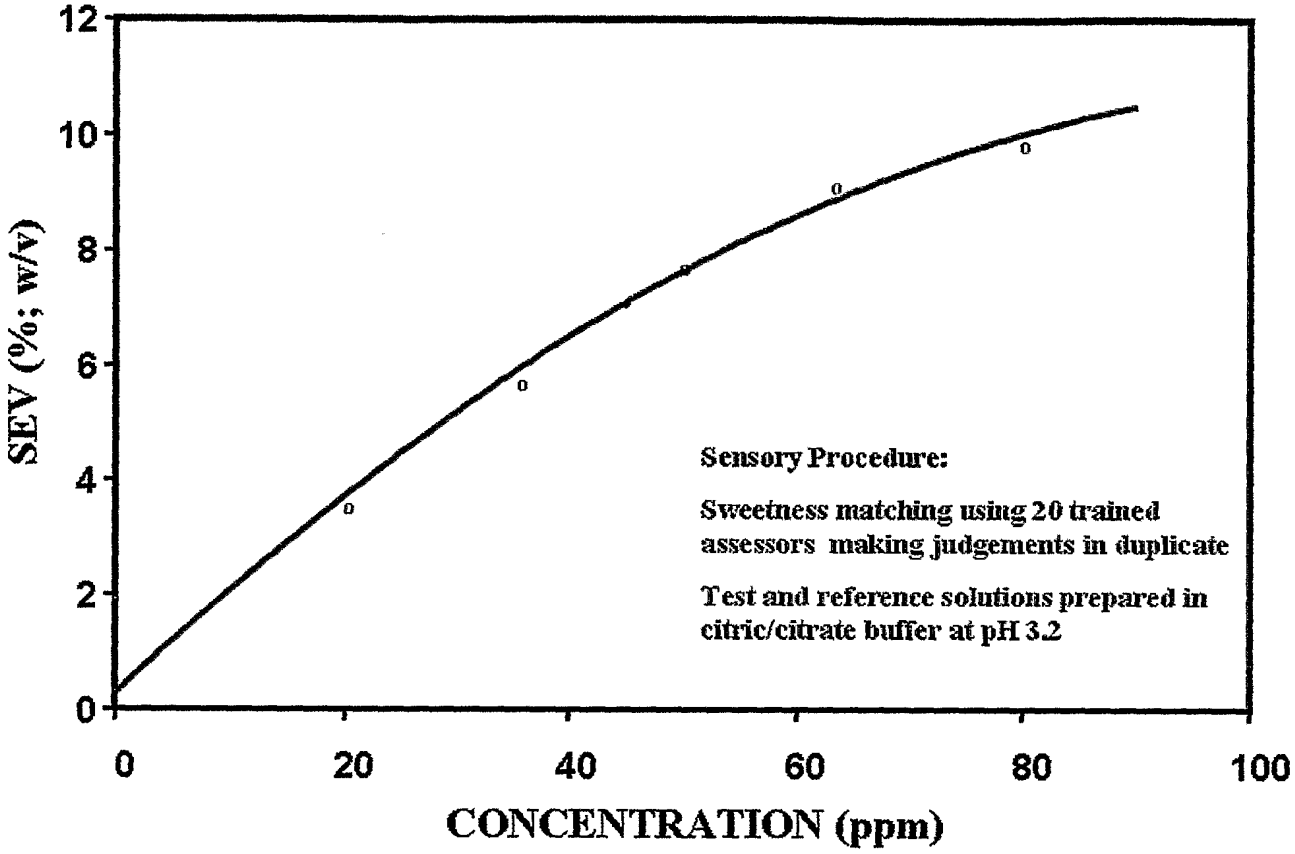

12
10
8
6
4
2
0
SEV (%; w/v)
0
20
40
60
80
100
CONCENTRATION (ppm)
Sensory Procedure:
Sweetness matching using 20 trained assessors making judgements in duplicate
Test and reference solutions prepared in citric/citrate buffer at pH 3.2

FIG. 16

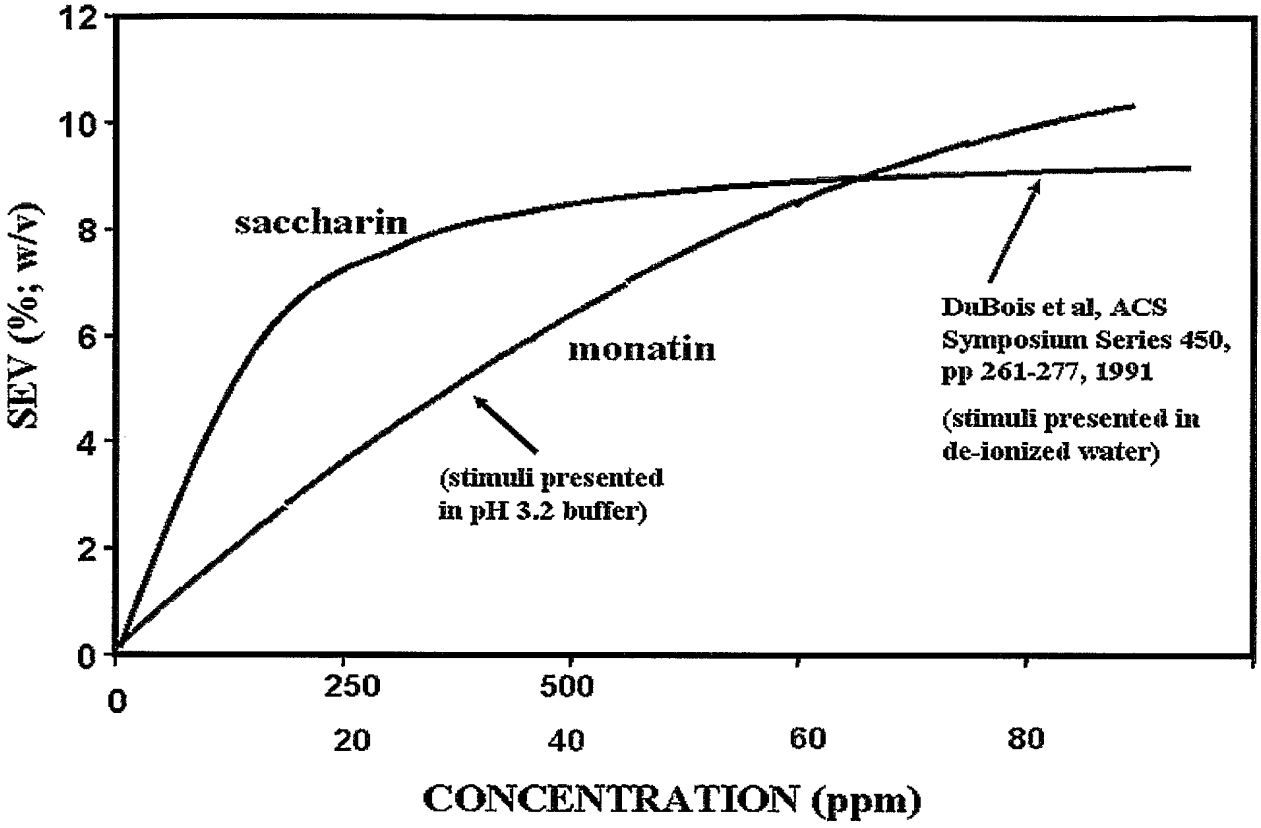

12
10
8
6
4
2
0
SEV (%; w/v)
saccharin
monatin
(stimuli presented in pH 3.2 buffer)
DuBois et al, ACS Symposium Series 450, pp 261-277, 1991
(stimuli presented in de-ionized water)
0
250
500
20
40
60
80
CONCENTRATION (ppm)

FIG. 17

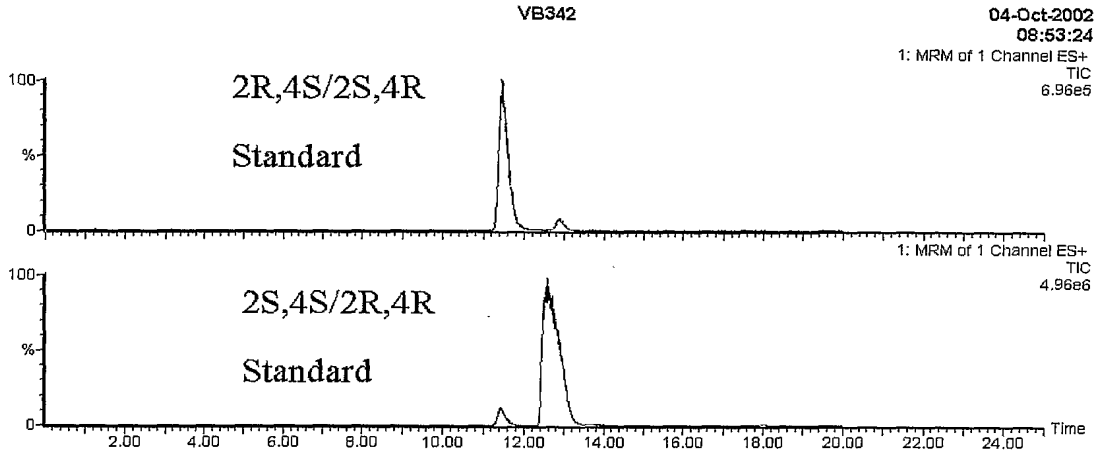
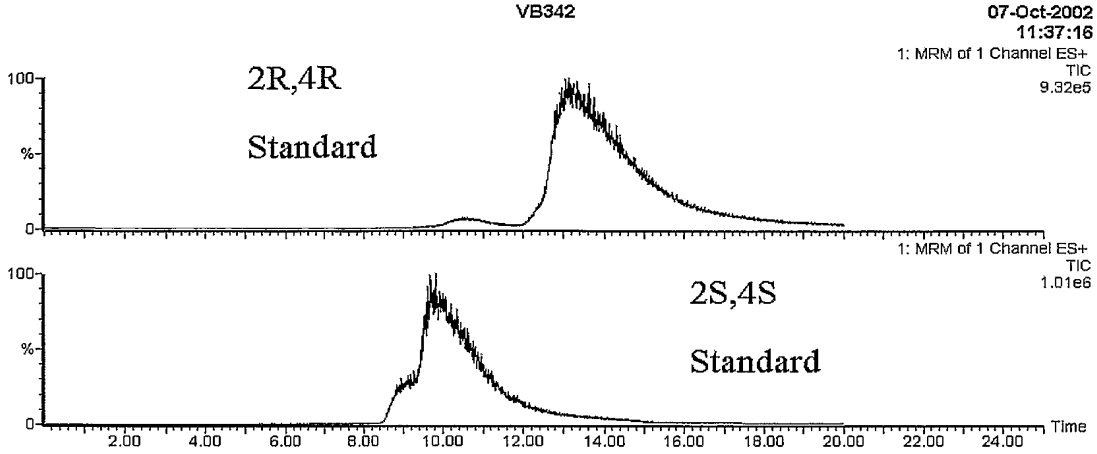

VB342
04-Oct-2002
08:53:24
1: MRM of 1 Channel ES+
TIC
6.96e5
100
%
0
2R,4S/2S,4R
Standard
1: MRM of 1 Channel ES+
TIC
4.96e6
100
%
0
2S,4S/2R,4R
Standard
Time
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00

FIG. 18


VB342
07-Oct-2002
11:37:16
1: MRM of 1 Channel ES+
TIC
9.32e5
2R,4R
Standard
100
%
0
1: MRM of 1 Channel ES+
TIC
1.01e6
2S,4S
Standard
100
%
0
Time
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00

SEQUENCE LISTING

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<120> Polypeptides and Biosynthetic Pathways

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<170> PatentIn version 3.1

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<213> Sinorhizobium meliloti

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aggaaggatg agcgccccgg aaaggtcgat ctcggcgtag gagtctatcg cgacgagacc 120

ggacgcacgc cgatcttccg ggccgtcaag gcggcggaaa agcggcttct cgaaacacag 180

gacagcaagg cctatatcgg ccccgaaggg gacctcgtct ttctcgatcg gctctgggaa 240

ctcgtcggcg gcgacacgat cgagcggagc catgttgcgg gcgtccagac gcccggcggc 300

tccggcgcgc tccgtttggc ggcggacctc atcgcccgca tgggcggccg aggcatctgg 360

ctcgggctgc cgagctggcc gaaccacgcg ccgatcttca aggcggccgg gctcgatatc 420

gccacctacg acttcttcga cattccgtcg cagtcggtca tcttcgataa tctggtgagc 480

gcgctggaag gcgccgcatc cggcgatgcg gtgctgctgc atgcaagctg ccacaacccg 540

accggcggcg tcctgagcga agcacaatgg atggagatcg ccgcgctggt ggccgagcgc 600

ggcctgctgc cgctcgtcga tctcgcctat caggggttcg gccgcggcct cgaccaggat 660

gtcgcgggcc tccggcatct tctcggcgtg gtcccggaag cgctcgtcgc ggtttcctgc 720

tcgaagtcct tcgggcttta tcgcgagcgc gcgggcgcga tcttcgcgcg gaccagctcg 780

actgcctcgg cggacagggt gcgctcaaac ctcgcgggcc tcgcacgcac cagctattcc 840

atgccgccgg atcacggcgc agccgtcgtg cggacgatcc ttgacgaccc ggaactcagg 900

cgcgactgga cggaggagct cgagacgatg cggctcagga tgacgggcct ccggcggtcg 960

cttgccgagg gactccgcac ccgctggcag agcctcggcg cagtcgccga tcaggagggc 1020

atgttctcca tgctgccgct ttccgaagcg gaggttatgc ggctcaggac cgagcacggc 1080

atctatatgc cggcatccgg ccgcatcaac atcgccgggc tgaagacggc ggaagccgcc 1140

gagattgccg gcaagttcac cagtctctga 1170

<210> 2
<211> 389
<212> PRT
<213> Sinorhizobium meliloti

<400> 2

Met Phe Asp Ala Leu Ala Arg Gln Ala Asp Asp Pro Leu Leu Phe Leu
1 5 10 15

Ile Gly Leu Phe Arg Lys Asp Glu Arg Pro Gly Lys Val Asp Leu Gly
20 25 30

Val Gly Val Tyr Arg Asp Glu Thr Gly Arg Thr Pro Ile Phe Arg Ala
35 40 45

Val Lys Ala Ala Glu Lys Arg Leu Leu Glu Thr Gln Asp Ser Lys Ala
50 55 60

Tyr Ile Gly Pro Glu Gly Asp Leu Val Phe Leu Asp Arg Leu Trp Glu
65 70 75 80

Leu Val Gly Gly Asp Thr Ile Glu Arg Ser His Val Ala Gly Val Gln
85 90 95

Thr Pro Gly Gly Ser Gly Ala Leu Arg Leu Ala Ala Asp Leu Ile Ala
100 105 110

Arg Met Gly Gly Arg Gly Ile Trp Leu Gly Leu Pro Ser Trp Pro Asn
115 120 125

His Ala Pro Ile Phe Lys Ala Ala Gly Leu Asp Ile Ala Thr Tyr Asp
130 135 140

Phe Phe Asp Ile Pro Ser Gln Ser Val Ile Phe Asp Asn Leu Val Ser
145 150 155 160

Ala Leu Glu Gly Ala Ala Ser Gly Asp Ala Val Leu Leu His Ala Ser
165 170 175

Cys His Asn Pro Thr Gly Gly Val Leu Ser Glu Ala Gln Trp Met Glu
180 185 190

Ile Ala Ala Leu Val Ala Glu Arg Gly Leu Leu Pro Leu Val Asp Leu
195 200 205

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Ala Tyr Gln Gly Phe Gly Arg Gly Leu Asp Gln Asp Val Ala Gly Leu
    210                 215                 220

Arg His Leu Leu Gly Val Val Pro Glu Ala Leu Val Ala Val Ser Cys
225                 230                 235                 240

Ser Lys Ser Phe Gly Leu Tyr Arg Glu Arg Ala Gly Ala Ile Phe Ala
                245                 250                 255

Arg Thr Ser Ser Thr Ala Ser Ala Asp Arg Val Arg Ser Asn Leu Ala
            260                 265                 270

Gly Leu Ala Arg Thr Ser Tyr Ser Met Pro Pro Asp His Gly Ala Ala
        275                 280                 285

Val Val Arg Thr Ile Leu Asp Asp Pro Glu Leu Arg Arg Asp Trp Thr
    290                 295                 300

Glu Glu Leu Glu Thr Met Arg Leu Arg Met Thr Gly Leu Arg Arg Ser
305                 310                 315                 320

Leu Ala Glu Gly Leu Arg Thr Arg Trp Gln Ser Leu Gly Ala Val Ala
                325                 330                 335

Asp Gln Glu Gly Met Phe Ser Met Leu Pro Leu Ser Glu Ala Glu Val
            340                 345                 350

Met Arg Leu Arg Thr Glu His Gly Ile Tyr Met Pro Ala Ser Gly Arg
        355                 360                 365

Ile Asn Ile Ala Gly Leu Lys Thr Ala Glu Ala Ala Glu Ile Ala Gly
    370                 375                 380

Lys Phe Thr Ser Leu
385
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<210> 3
<211> 1260
<212> DNA
<213> Rhodobacter sphaeroides

<400> 3

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aaggatgacg aaggaatgct gaccgccctg aagccgcagc ccgcggacaa gatcctgcaa    120

ctgatccaga tgttccgcga ggatgcgcgc gcggacaaga tcgatctggg cgtgggcgtc    180

tacaaggacc cgaccgggct caccccggtc atgcgggccg tgaaggcggc cgagaagcgg    240
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ctctgggagg tcgagaccac caagacctac accggccttg ccgacgagcc ggcctacaat 300

gccgcgatgg cgaagctgat cctcgcgggc gcggtcccgg ccgaccgggt ggcctcggtc 360

gccacccccg gcggcacggg cgcggtgcgt caggcgctcg agctgatccg catggcctcg 420

cccgaggcca ccgtctggat ctcgaacccg acctggccga accatctgtc gatcgtgaaa 480

tatctcggca tcccgatgcg ggaataccgc tatttcgacg ccgagaccgg cgccgtcgat 540

gccgagggca tgatggagga tctggcccag gtgaaggcgg gcgacgtggt gctgctgcac 600

ggctgctgcc acaacccgac cggcgccaac ccgaacccgg tgcagtggct ggccatctgc 660

gagagcctgg cccggacagg cgcggtgccg ctgatcgacc tcgcctatca gggcttcggc 720

gacgggctcg agatggatgc ggcggcgacg cggcttctgg ccaccagact gcccgaggtg 780

ctgatcgcgg cctcctgctc gaagaacttc ggcatctacc gcgagcgcac gggcatcctg 840

atcgccatcg gcgaggcggc gggccggggc acggtgcagg ccaacctcaa cttcctgaac 900

cggcagaact actccttccc gccggaccat ggcgcgcggc tcgtgaccat gatcctcgag 960

gacgagacgc tgagcgccga ctggaaggcg gaactcgagg aggtgcggct caacatgctg 1020

acactgcgcc gccagcttgc cgatgcgctg caggccgaga ccggctcgaa ccgcttcggc 1080

ttcgtggccg agcatcgcgg catgttctcg cgcctcggga tcacgcccgc cgaggtggag 1140

cggctgcgga ccgagcacgg ggtctacatg gtgggcgatt cgcggctgaa catcgcgggg 1200

ctgaaccgga cgaccgtgcc ggtgctggcg cgcgcggtgg ccaaggtgct gcgcggctga 1260

<210> 4

<211> 419
<212> PRT
<213> Rhodobacter sphaeroides

<400> 4

Met Arg Ser Thr Thr Ala Pro Gly Pro Ser Gly Ala Cys Met Thr Ile
1 5 10 15

Ser Arg Ser Arg Lys Asp Asp Glu Gly Met Leu Thr Ala Leu Lys Pro
20 25 30

Gln Pro Ala Asp Lys Ile Leu Gln Leu Ile Gln Met Phe Arg Glu Asp
35 40 45

Ala Arg Ala Asp Lys Ile Asp Leu Gly Val Gly Val Tyr Lys Asp Pro
50 55 60

Thr Gly Leu Thr Pro Val Met Arg Ala Val Lys Ala Ala Glu Lys Arg

65 70 75 80

Leu Trp Glu Val Glu Thr Thr Lys Thr Tyr Thr Gly Leu Ala Asp Glu
85 90 95

Pro Ala Tyr Asn Ala Ala Met Ala Lys Leu Ile Leu Ala Gly Ala Val
100 105 110

Pro Ala Asp Arg Val Ala Ser Val Ala Thr Pro Gly Gly Thr Gly Ala
115 120 125

Val Arg Gln Ala Leu Glu Leu Ile Arg Met Ala Ser Pro Glu Ala Thr
130 135 140

Val Trp Ile Ser Asn Pro Thr Trp Pro Asn His Leu Ser Ile Val Lys
145 150 155 160

Tyr Leu Gly Ile Pro Met Arg Glu Tyr Arg Tyr Phe Asp Ala Glu Thr
165 170 175

Gly Ala Val Asp Ala Glu Gly Met Met Glu Asp Leu Ala Gln Val Lys
180 185 190

Ala Gly Asp Val Val Leu Leu His Gly Cys Cys His Asn Pro Thr Gly
195 200 205

Ala Asn Pro Asn Pro Val Gln Trp Leu Ala Ile Cys Glu Ser Leu Ala
210 215 220

Arg Thr Gly Ala Val Pro Leu Ile Asp Leu Ala Tyr Gln Gly Phe Gly
225 230 235 240

Asp Gly Leu Glu Met Asp Ala Ala Ala Thr Arg Leu Leu Ala Thr Arg
245 250 255

Leu Pro Glu Val Leu Ile Ala Ala Ser Cys Ser Lys Asn Phe Gly Ile
260 265 270

Tyr Arg Glu Arg Thr Gly Ile Leu Ile Ala Ile Gly Glu Ala Ala Gly
275 280 285

Arg Gly Thr Val Gln Ala Asn Leu Asn Phe Leu Asn Arg Gln Asn Tyr
290 295 300

Ser Phe Pro Pro Asp His Gly Ala Arg Leu Val Thr Met Ile Leu Glu
305 310 315 320

Asp Glu Thr Leu Ser Ala Asp Trp Lys Ala Glu Leu Glu Glu Val Arg
325 330 335

Leu Asn Met Leu Thr Leu Arg Arg Gln Leu Ala Asp Ala Leu Gln Ala
340 345 350

Glu Thr Gly Ser Asn Arg Phe Gly Phe Val Ala Glu His Arg Gly Met
355 360 365

Phe Ser Arg Leu Gly Ile Thr Pro Ala Glu Val Glu Arg Leu Arg Thr
370 375 380

Glu His Gly Val Tyr Met Val Gly Asp Ser Arg Leu Asn Ile Ala Gly
385 390 395 400

Leu Asn Arg Thr Thr Val Pro Val Leu Ala Arg Ala Val Ala Lys Val
405 410 415

Leu Arg Gly

<210> 5
<211> 1260
<212> DNA
<213> Rhodobacter sphaeroides

<400> 5
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aaggatgacg aaggaatgct gaccgccctg aagccgcagc ccgcggacaa gatcctgcaa 120

ctgatccaga tgttccgcga ggatgcgcgc gcggacaaga tcgatctggg cgtgggcgtc 180

tacaaggacc cgaccgggct caccccggtc atgcgggccg tgaaggccgc cgagaagcgg 240

ctctgggagg tcgagaccac caagacctac accggccttg ccggcgagcc cgcctacaat 300

gccgcgatgg cgaagctgat cctcgcaggc gcggtcccgg ccgaccgggt ggcctcggtc 360

gccacccccg gcggcacggg cgcggtgcgt caggcgctcg agctgatccg catggcctcg 420

cccgaggcca ctgtctggat ctcgaacccg acctggccga accatctgtc gatcgtgaaa 480

tatctcggca tcccgatgcg ggaataccgc tatttcgacg ccgagaccgg cgccgtcgat 540

gccgagggct tgatggagga tctggcccag gtgaaggcgg gcgacgtggt gctgctgcac 600

ggctgctgcc acaacccgac cggcgccaac ccgaacccgg tgcagtggct ggccgtctgc 660

gagagcctgg cccggacagg cgcggtgccg ctgatcgacc tcgcctatca gggcttcggc 720

gacgggctcg agatggatgc ggcggcgacg cggcttctgg ccaccagact gcccgaggtg 780

ctgatcgcgg cctcctgctc gaagaacttc ggcatctacc gcgagcgaac gggcatcctg 840

atcgccatcg gcgaggcggc gggccggggc acggtgcagg ccaacctcaa cttcctgaac 900

cggcagaact actccttccc gccggaccat ggcgcgcggc tcgtgaccat gatcctcgag 960

gacgagacgc tgagcgccga ctggaaggcg gaactcgagg aggtgcggct caacatgctg 1020

acgctgcgcc gccagcttgc cgatgcgctg caggccgaga ccggctcgaa ccgcttcggc 1080

ttcgtggccg agcatcgcgg catgttctcg cgcctcggga tcacgcccgc cgaggtggag 1140

cggctgcgga ccgagcacgg ggtctacatg gtgggcgatt cgcggctgaa catcgcgggg 1200

ctgaaccgga cgaccgtgcc ggtgctggcg cgcgcggtgg ccaaggtgct gcgcggctga 1260

<210> 6
<211> 419
<212> PRT
<213> Rhodobacter sphaeroides

<400> 6

Met Arg Ser Thr Thr Ala Pro Gly Pro Ser Gly Ala Cys Met Thr Ile
1 5 10 15

Ser Arg Ser Arg Lys Asp Asp Glu Gly Met Leu Thr Ala Leu Lys Pro
20 25 30

Gln Pro Ala Asp Lys Ile Leu Gln Leu Ile Gln Met Phe Arg Glu Asp
35 40 45

Ala Arg Ala Asp Lys Ile Asp Leu Gly Val Gly Val Tyr Lys Asp Pro
50 55 60

Thr Gly Leu Thr Pro Val Met Arg Ala Val Lys Ala Ala Glu Lys Arg
65 70 75 80

Leu Trp Glu Val Glu Thr Thr Lys Thr Tyr Thr Gly Leu Ala Gly Glu
85 90 95

Pro Ala Tyr Asn Ala Ala Met Ala Lys Leu Ile Leu Ala Gly Ala Val
100 105 110

Pro Ala Asp Arg Val Ala Ser Val Ala Thr Pro Gly Gly Thr Gly Ala
115 120 125

Val Arg Gln Ala Leu Glu Leu Ile Arg Met Ala Ser Pro Glu Ala Thr
130 135 140

```
Val Trp Ile Ser Asn Pro Thr Trp Pro Asn His Leu Ser Ile Val Lys
145                 150                 155                 160

Tyr Leu Gly Ile Pro Met Arg Glu Tyr Arg Tyr Phe Asp Ala Glu Thr
                165                 170                 175

Gly Ala Val Asp Ala Glu Gly Leu Met Glu Asp Leu Ala Gln Val Lys
            180                 185                 190

Ala Gly Asp Val Val Leu Leu His Gly Cys Cys His Asn Pro Thr Gly
        195                 200                 205

Ala Asn Pro Asn Pro Val Gln Trp Leu Ala Val Cys Glu Ser Leu Ala
    210                 215                 220

Arg Thr Gly Ala Val Pro Leu Ile Asp Leu Ala Tyr Gln Gly Phe Gly
225                 230                 235                 240

Asp Gly Leu Glu Met Asp Ala Ala Ala Thr Arg Leu Leu Ala Thr Arg
                245                 250                 255

Leu Pro Glu Val Leu Ile Ala Ala Ser Cys Ser Lys Asn Phe Gly Ile
            260                 265                 270

Tyr Arg Glu Arg Thr Gly Ile Leu Ile Ala Ile Gly Glu Ala Ala Gly
        275                 280                 285

Arg Gly Thr Val Gln Ala Asn Leu Asn Phe Leu Asn Arg Gln Asn Tyr
    290                 295                 300

Ser Phe Pro Pro Asp His Gly Ala Arg Leu Val Thr Met Ile Leu Glu
305                 310                 315                 320

Asp Glu Thr Leu Ser Ala Asp Trp Lys Ala Glu Leu Glu Glu Val Arg
                325                 330                 335

Leu Asn Met Leu Thr Leu Arg Arg Gln Leu Ala Asp Ala Leu Gln Ala
            340                 345                 350

Glu Thr Gly Ser Asn Arg Phe Gly Phe Val Ala Glu His Arg Gly Met
        355                 360                 365

Phe Ser Arg Leu Gly Ile Thr Pro Ala Glu Val Glu Arg Leu Arg Thr
    370                 375                 380

Glu His Gly Val Tyr Met Val Gly Asp Ser Arg Leu Asn Ile Ala Gly
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385 390 395 400

Leu Asn Arg Thr Thr Val Pro Val Leu Ala Arg Ala Val Ala Lys Val
405 410 415

Leu Arg Gly

<210> 7
<211> 1239
<212> DNA
<213> Leishmania major

<400> 7
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cccgatgtca tcttcgatct cgcaaaacgc gccgccgctg ccaagggccc caaggccaac 120

ctcgtcattg gtgcctaccg cgacgagcag ggccgtccct atccgctacg cgtggtccgc 180

aaggctgagc agcttctctt ggacatgaat ctcgactacg agtacctacc catctcgggc 240

taccagccct tcatcgatga ggcggtaaag attatctacg gcaataccgt cgagctggag 300

aacctggttg cggtgcagac gctgagcggg accggtgctg tctctctcgg ggcgaagctg 360

ctgactcgcg tcttcgacgc tgagacgacg cccatctacc tttccgaccc cacgtggccc 420

aaccactacg gcgtcgtgaa ggctgctggc tggaagaaca tctgcacgta cgcctactac 480

gaccccaaga cggtcagcct gaatttcgag ggcatgaaga aagacattct ggcggcgccg 540

gacggctccg tgttcattct gcaccagtgc gcgcacaacc ccaccggcgt ggacccgtcg 600

caggagcagt ggaacgagat cgcgtcactg atgctggcca agcaccatca ggtgttcttc 660

gactccgcct accaaggcta tgcgagcggc agcctcgaca cggacgcgta tgctgcccgc 720

ctgtttgccc gccgcggcat cgaggtactg ctggcgcagt cgttctccaa gaacatgggc 780

ttgtacagcg agcgtgcagg cacgctgtcg ctgctcctca aggacaagac gaagcgcgcg 840

gatgtaaaga gcgtgatgga ttcgctgatc cgtgaggagt acacgtgccc cccagcccac 900

ggtgcccgct tagcccacct aatcctgagc aacaacgaac tgcgaaagga gtgggaggca 960

gagctatcag ccatggcaga gcgcatccgt acgatgcgcc gcaccgtgta cgacgagctg 1020

ctgcgcctgc agacgcccgg gagctgggaa catgtcatta accagattgg catgttttcc 1080

ttcctcgggc tgtcaaaggc gcagtgcgaa tactgccaaa accacaacat cttcatcaca 1140

gtgtcgggcc gcgctaacat ggcaggtctg acgcatgaga cggcgctgat gctagcacag 1200

acgatcaacg atgctgtgcg caatgtgaat cgtgagtga 1239

<210> 8

<211> 412
<212> PRT
<213> Leishmania major

<400> 8

Met Ser Met Gln Ala Ala Met Thr Thr Ala Glu Arg Trp Gln Lys Ile
1 5 10 15

Gln Ala Gln Ala Pro Asp Val Ile Phe Asp Leu Ala Lys Arg Ala Ala
20 25 30

Ala Ala Lys Gly Pro Lys Ala Asn Leu Val Ile Gly Ala Tyr Arg Asp
35 40 45

Glu Gln Gly Arg Pro Tyr Pro Leu Arg Val Val Arg Lys Ala Glu Gln
50 55 60

Leu Leu Leu Asp Met Asn Leu Asp Tyr Glu Tyr Leu Pro Ile Ser Gly
65 70 75 80

Tyr Gln Pro Phe Ile Asp Glu Ala Val Lys Ile Ile Tyr Gly Asn Thr
85 90 95

Val Glu Leu Glu Asn Leu Val Ala Val Gln Thr Leu Ser Gly Thr Gly
100 105 110

Ala Val Ser Leu Gly Ala Lys Leu Leu Thr Arg Val Phe Asp Ala Glu
115 120 125

Thr Thr Pro Ile Tyr Leu Ser Asp Pro Thr Trp Pro Asn His Tyr Gly
130 135 140

Val Val Lys Ala Ala Gly Trp Lys Asn Ile Cys Thr Tyr Ala Tyr Tyr
145 150 155 160

Asp Pro Lys Thr Val Ser Leu Asn Phe Glu Gly Met Lys Lys Asp Ile
165 170 175

Leu Ala Ala Pro Asp Gly Ser Val Phe Ile Leu His Gln Cys Ala His
180 185 190

Asn Pro Thr Gly Val Asp Pro Ser Gln Glu Gln Trp Asn Glu Ile Ala
195 200 205

Ser Leu Met Leu Ala Lys His His Gln Val Phe Phe Asp Ser Ala Tyr
210 215 220

```
Gln Gly Tyr Ala Ser Gly Ser Leu Asp Thr Asp Ala Tyr Ala Ala Arg
225                 230                 235                 240

Leu Phe Ala Arg Arg Gly Ile Glu Val Leu Leu Ala Gln Ser Phe Ser
                245                 250                 255

Lys Asn Met Gly Leu Tyr Ser Glu Arg Ala Gly Thr Leu Ser Leu Leu
            260                 265                 270

Leu Lys Asp Lys Thr Lys Arg Ala Asp Val Lys Ser Val Met Asp Ser
        275                 280                 285

Leu Ile Arg Glu Glu Tyr Thr Cys Pro Pro Ala His Gly Ala Arg Leu
    290                 295                 300

Ala His Leu Ile Leu Ser Asn Asn Glu Leu Arg Lys Glu Trp Glu Ala
305                 310                 315                 320

Glu Leu Ser Ala Met Ala Glu Arg Ile Arg Thr Met Arg Arg Thr Val
                325                 330                 335

Tyr Asp Glu Leu Leu Arg Leu Gln Thr Pro Gly Ser Trp Glu His Val
            340                 345                 350

Ile Asn Gln Ile Gly Met Phe Ser Phe Leu Gly Leu Ser Lys Ala Gln
        355                 360                 365

Cys Glu Tyr Cys Gln Asn His Asn Ile Phe Ile Thr Val Ser Gly Arg
    370                 375                 380

Ala Asn Met Ala Gly Leu Thr His Glu Thr Ala Leu Met Leu Ala Gln
385                 390                 395                 400

Thr Ile Asn Asp Ala Val Arg Asn Val Asn Arg Glu
                405                 410
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<210> 9
<211> 1182
<212> DNA
<213> Bacillus subtilis

<400> 9
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tcgaatcttg tagcccaaca cgaagacgtc atttcactta caatcggcca gcctgatttt 120

ttcacaccgc atcatgtgaa agctgccgca aaaaaagcca ttgatgaaaa cgtgacgtca 180

tatactccga atgccggcta cctggagctg agacaagctg tgcagcttta tatgaagaaa 240

aaagcggatt tcaactatga tgctgaatct gaaattatca tcacaacagg cgcaagccaa 300

gccattgatg ctgcattccg gacgatttta tctcccggtg atgaagtcat tatgccaggg 360

cctatttatc cgggctatga acctattatc aatttgtgcg gggccaagcc tgtcattgtt 420

gatactacgt cacacggctt taagcttacc gcccggctga ttgaagatgc tctgacaccc 480

aacaccaagt gtgtcgtgct tccttatccg tcaaacccta ccggcgtgac tttatctgaa 540

gaagaactga aaagcatcgc agctctctta aaaggcagaa atgtcttcgt attgtctgat 600

gaaatataca gtgaattaac atatgacaga ccgcattact ccatcgcaac ctatttgcgg 660

gatcaaacga ttgtcattaa cgggttgtca aaatcacaca gcatgaccgg ttggagaatt 720

ggatttttat ttgcaccgaa agacattgca aagcacattt taaaggttca tcaatacaat 780

gtgtcgtgcg cctcatccat ttctcaaaaa gccgcgcttg aagctgtcac aaacggcttt 840

gacgatgcat tgattatgag agaacaatac aaaaaacgtc tggactatgt ttatgaccgt 900

cttgtttcca tgggacttga cgtagttaaa ccgtccggtg cgttttatat cttcccttct 960

attaaatcat ttggaatgac ttcatttgat tttagtatgg ctcttttgga agacgctggc 1020

gtggcactcg tgccgggcag ctcgttctca acatatggtg aaggatatgt aaggctgtct 1080

tttgcatgct caatggacac gctgagagaa ggcctagacc gtttagaatt atttgtatta 1140

aaaaaacgtg aagcaatgca gacgataaac aacggcgttt aa 1182

<210> 10
<211> 393
<212> PRT
<213> Bacillus subtilis

<400> 10

Met Glu His Leu Leu Asn Pro Lys Ala Arg Glu Ile Glu Ile Ser Gly
1 5 10 15

Ile Arg Lys Phe Ser Asn Leu Val Ala Gln His Glu Asp Val Ile Ser
20 25 30

Leu Thr Ile Gly Gln Pro Asp Phe Phe Thr Pro His His Val Lys Ala
35 40 45

Ala Ala Lys Lys Ala Ile Asp Glu Asn Val Thr Ser Tyr Thr Pro Asn
50 55 60

Ala Gly Tyr Leu Glu Leu Arg Gln Ala Val Gln Leu Tyr Met Lys Lys
65 70 75 80

Lys Ala Asp Phe Asn Tyr Asp Ala Glu Ser Glu Ile Ile Ile Thr Thr
85 90 95

Gly Ala Ser Gln Ala Ile Asp Ala Ala Phe Arg Thr Ile Leu Ser Pro
100 105 110

Gly Asp Glu Val Ile Met Pro Gly Pro Ile Tyr Pro Gly Tyr Glu Pro
115 120 125

Ile Ile Asn Leu Cys Gly Ala Lys Pro Val Ile Val Asp Thr Thr Ser
130 135 140

His Gly Phe Lys Leu Thr Ala Arg Leu Ile Glu Asp Ala Leu Thr Pro
145 150 155 160

Asn Thr Lys Cys Val Val Leu Pro Tyr Pro Ser Asn Pro Thr Gly Val
165 170 175

Thr Leu Ser Glu Glu Glu Leu Lys Ser Ile Ala Ala Leu Leu Lys Gly
180 185 190

Arg Asn Val Phe Val Leu Ser Asp Glu Ile Tyr Ser Glu Leu Thr Tyr
195 200 205

Asp Arg Pro His Tyr Ser Ile Ala Thr Tyr Leu Arg Asp Gln Thr Ile
210 215 220

Val Ile Asn Gly Leu Ser Lys Ser His Ser Met Thr Gly Trp Arg Ile
225 230 235 240

Gly Phe Leu Phe Ala Pro Lys Asp Ile Ala Lys His Ile Leu Lys Val
245 250 255

His Gln Tyr Asn Val Ser Cys Ala Ser Ser Ile Ser Gln Lys Ala Ala
260 265 270

Leu Glu Ala Val Thr Asn Gly Phe Asp Asp Ala Leu Ile Met Arg Glu
275 280 285

Gln Tyr Lys Lys Arg Leu Asp Tyr Val Tyr Asp Arg Leu Val Ser Met
290 295 300

Gly Leu Asp Val Val Lys Pro Ser Gly Ala Phe Tyr Ile Phe Pro Ser
305 310 315 320

Ile Lys Ser Phe Gly Met Thr Ser Phe Asp Phe Ser Met Ala Leu Leu
325 330 335

Glu Asp Ala Gly Val Ala Leu Val Pro Gly Ser Ser Phe Ser Thr Tyr
340 345 350

Gly Glu Gly Tyr Val Arg Leu Ser Phe Ala Cys Ser Met Asp Thr Leu
355 360 365

Arg Glu Gly Leu Asp Arg Leu Glu Leu Phe Val Leu Lys Lys Arg Glu
370 375 380

Ala Met Gln Thr Ile Asn Asn Gly Val
385 390

<210> 11
<211> 1176
<212> DNA
<213> Lactobacillus amylovorus

<400> 11
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ggaattagaa tctttgataa caaagtttca gctattcctg gcattatcaa attgactttg 120

ggtgaaccag atatgaatac tcctgagcat gttaagcaag cggctattaa gaatattgca 180

gataatgatt cacactatgc tccacaaaag ggaaagcttg aattaagaaa agctatcagt 240

aaatatttga aaaagattac tggaattgaa tatgatccag aaacagaaat cgtagtaaca 300

gttggtgcaa ctgaagcaat taacgctacc ttgtttgcta ttactaatcc gggtgacaag 360

gttgcaattc ctacgccagt cttttctcta tattggcccg tggctacact tgctgatgcc 420

gattatgttt tgatgaatac tgcagaagat ggttttaagt taacacctaa gaagttagaa 480

gaaactatca aagaaaatcc aacaattaaa gcagtaattt tgaattatcc aactaaccca 540

actggtgttg aatatagcga agatgaaatt aaagctttgg ctaaggtaat taaagataat 600

catctgtacg taattaccga tgaaatttac agtactttga cttacggtgt aaaacacttt 660

tcaattgcca gcttaattcc agaaagagca atttatatct ctggtttatc taaatcacat 720

gcgatgactg gttatcgttt aggctatgtt gccggacctg caaaaattat ggcagaaatt 780

ggtaaagttc atggccttat ggtgacgact acgacggatt catcacaagc tgccgcaatt 840

gaagcacttg aacacggact tgatgaccct gagaaatata gggaagttta tgaaaagcgt 900

cgtgactatg ttttaaagga attagccgag atagagatgc aagcagttaa gccagaaggt 960

gcattttata tctttgctaa aattccagct aagtatggca aagacgatat gaaatttgcc 1020

ttggatttag cttttaaaga aaaagtgggt atcactccag gtagtgcatt tggtcctggt 1080

ggtgaaggtc atattagatt atcttatgca tcaagtgatg aaaacttgca tgaggcaatg 1140

aagcgaatga agaaagtttt acaagaggac gaataa 1176

<210> 12
<211> 391
<212> PRT
<213> Lactobacillus amylovorus

<400> 12

Met Pro Glu Leu Ala Asn Asp Leu Gly Leu Ser Lys Lys Ile Thr Asp
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Val Lys Ala Ser Gly Ile Arg Ile Phe Asp Asn Lys Val Ser Ala Ile
20 25 30

Pro Gly Ile Ile Lys Leu Thr Leu Gly Glu Pro Asp Met Asn Thr Pro
35 40 45

Glu His Val Lys Gln Ala Ala Ile Lys Asn Ile Ala Asp Asn Asp Ser
50 55 60

His Tyr Ala Pro Gln Lys Gly Lys Leu Glu Leu Arg Lys Ala Ile Ser
65 70 75 80

Lys Tyr Leu Lys Lys Ile Thr Gly Ile Glu Tyr Asp Pro Glu Thr Glu
85 90 95

Ile Val Val Thr Val Gly Ala Thr Glu Ala Ile Asn Ala Thr Leu Phe
100 105 110

Ala Ile Thr Asn Pro Gly Asp Lys Val Ala Ile Pro Thr Pro Val Phe
115 120 125

Ser Leu Tyr Trp Pro Val Ala Thr Leu Ala Asp Ala Asp Tyr Val Leu
130 135 140

Met Asn Thr Ala Glu Asp Gly Phe Lys Leu Thr Pro Lys Lys Leu Glu
145 150 155 160

Glu Thr Ile Lys Glu Asn Pro Thr Ile Lys Ala Val Ile Leu Asn Tyr
165 170 175

Pro Thr Asn Pro Thr Gly Val Glu Tyr Ser Glu Asp Glu Ile Lys Ala
180 185 190

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Leu Ala Lys Val Ile Lys Asp Asn His Leu Tyr Val Ile Thr Asp Glu
        195                 200                 205

Ile Tyr Ser Thr Leu Thr Tyr Gly Val Lys His Phe Ser Ile Ala Ser
    210                 215                 220

Leu Ile Pro Glu Arg Ala Ile Tyr Ile Ser Gly Leu Ser Lys Ser His
225                 230                 235                 240

Ala Met Thr Gly Tyr Arg Leu Gly Tyr Val Ala Gly Pro Ala Lys Ile
                245                 250                 255

Met Ala Glu Ile Gly Lys Val His Gly Leu Met Val Thr Thr Thr Thr
            260                 265                 270

Asp Ser Ser Gln Ala Ala Ala Ile Glu Ala Leu Glu His Gly Leu Asp
        275                 280                 285

Asp Pro Glu Lys Tyr Arg Glu Val Tyr Glu Lys Arg Arg Asp Tyr Val
    290                 295                 300

Leu Lys Glu Leu Ala Glu Ile Glu Met Gln Ala Val Lys Pro Glu Gly
305                 310                 315                 320

Ala Phe Tyr Ile Phe Ala Lys Ile Pro Ala Lys Tyr Gly Lys Asp Asp
                325                 330                 335

Met Lys Phe Ala Leu Asp Leu Ala Phe Lys Glu Lys Val Gly Ile Thr
            340                 345                 350

Pro Gly Ser Ala Phe Gly Pro Gly Gly Glu Gly His Ile Arg Leu Ser
        355                 360                 365

Tyr Ala Ser Ser Asp Glu Asn Leu His Glu Ala Met Lys Arg Met Lys
    370                 375                 380

Lys Val Leu Gln Glu Asp Glu
385                 390
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<210> 13
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<212> DNA
<213> R. sphaeroides

<400> 13
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atcgccgagg cgatcaccct cgacatcacc cgcgggcggc tgaggcccgg agcgagactg 120

cccggcacac gcgcgctggc gcgggcgctc ggcgtgcatc gcaacacggt ggatgccgcc 180

tatcaggagt tgctgaccca gggctggctg caggccgagc ccgcgcgggg caccttcgtg 240

gcgcaggatc tgccgcaggg gatgctggtg cacaggcccg cgcccgcgcc ggtcgagccg 300

gtcgcgatgc gcgcggggct cgccttctcc gatggcgcgc cggaccccga gctggtgccc 360

gacaaggcgc tggcgcgggc ctttcgccgg gcgctcctgt cgcccgcctt ccgcgccgga 420

gcggattacg gcgatgcccg cggcacctcc tcgctgcggg aggcgctggc agcctatctc 480

gcctcggacc ggggcgtggt cgcggatcct gcgcggctgc tgatcgcgcg gggcagccag 540

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gagccgggct atccgctggc ctgggaggcg ttccgcgcag cgggagcgga ggtgcgcggc 660

gtgccggtgg atggcggcgg cctcaggatc gacgcgctcg aggccgcgct ggcccgggat 720

ccgcgaatcc gggcggtcta tgtcacgccc catcaccagt atccgacgac cgtcaccatg 780

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gaggacgact acgaccacga ataccgcttc gagggccgtc cggtgctgcc gctggctgcc 900

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cgcgccgcca tcgaccggca gggcgacgcg ccgctcgagg cggcgctggc cgagctgatc 1080

cgcgacggcg atctgggccg tcatgcccgc aaggcgcgca gggtctaccg ggcgcggcgg 1140

gatctgctgg cggagcgtct cacggcgcag ctggccgggc gcgccgcctt cgatctgccg 1200

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gtggagatcc tcgcccggag cttccccggc tga 1413

<210> 14
<211> 470
<212> PRT
<213> R. sphaeroides

<400> 14

Met Arg Glu Pro Leu Ala Leu Glu Ile Asp Pro Gly His Gly Gly Pro
1 5 10 15

Leu Phe Leu Ala Ile Ala Glu Ala Ile Thr Leu Asp Ile Thr Arg Gly
20 25 30

Arg Leu Arg Pro Gly Ala Arg Leu Pro Gly Thr Arg Ala Leu Ala Arg
35 40 45

Ala Leu Gly Val His Arg Asn Thr Val Asp Ala Ala Tyr Gln Glu Leu
50 55 60

Leu Thr Gln Gly Trp Leu Gln Ala Glu Pro Ala Arg Gly Thr Phe Val
65 70 75 80

Ala Gln Asp Leu Pro Gln Gly Met Leu Val His Arg Pro Ala Pro Ala
85 90 95

Pro Val Glu Pro Val Ala Met Arg Ala Gly Leu Ala Phe Ser Asp Gly
100 105 110

Ala Pro Asp Pro Glu Leu Val Pro Asp Lys Ala Leu Ala Arg Ala Phe
115 120 125

Arg Arg Ala Leu Leu Ser Pro Ala Phe Arg Ala Gly Ala Asp Tyr Gly
130 135 140

Asp Ala Arg Gly Thr Ser Ser Leu Arg Glu Ala Leu Ala Ala Tyr Leu
145 150 155 160

Ala Ser Asp Arg Gly Val Val Ala Asp Pro Ala Arg Leu Leu Ile Ala
165 170 175

Arg Gly Ser Gln Met Ala Leu Phe Leu Val Ala Arg Ala Ala Leu Ala
180 185 190

Pro Gly Glu Ala Ile Ala Val Glu Glu Pro Gly Tyr Pro Leu Ala Trp
195 200 205

Glu Ala Phe Arg Ala Ala Gly Ala Glu Val Arg Gly Val Pro Val Asp
210 215 220

Gly Gly Gly Leu Arg Ile Asp Ala Leu Glu Ala Ala Leu Ala Arg Asp
225 230 235 240

Pro Arg Ile Arg Ala Val Tyr Val Thr Pro His His Gln Tyr Pro Thr
245 250 255

Thr Val Thr Met Gly Ala Ala Arg Arg Leu Gln Leu Leu Glu Leu Ala
260 265 270

Glu Arg His Arg Leu Ala Leu Ile Glu Asp Asp Tyr Asp His Glu Tyr
275 280 285

Arg Phe Glu Gly Arg Pro Val Leu Pro Leu Ala Ala Arg Ala Pro Glu
290 295 300

Gly Leu Pro Leu Ile Tyr Val Gly Ser Leu Ser Lys Leu Leu Ser Pro
305 310 315 320

Gly Ile Arg Leu Gly Tyr Ala Leu Ala Pro Glu Arg Leu Leu Thr Arg
325 330 335

Met Ala Ala Ala Arg Ala Ala Ile Asp Arg Gln Gly Asp Ala Pro Leu
340 345 350

Glu Ala Ala Leu Ala Glu Leu Ile Arg Asp Gly Asp Leu Gly Arg His
355 360 365

Ala Arg Lys Ala Arg Arg Val Tyr Arg Ala Arg Arg Asp Leu Leu Ala
370 375 380

Glu Arg Leu Thr Ala Gln Leu Ala Gly Arg Ala Ala Phe Asp Leu Pro
385 390 395 400

Ala Gly Gly Leu Ala Leu Trp Leu Arg Cys Ala Gly Val Ser Ala Glu
405 410 415

Thr Trp Ala Glu Ala Ala Gly Gln Ala Gly Leu Ala Leu Leu Pro Gly
420 425 430

Thr Arg Phe Ala Leu Glu Ser Pro Ala Pro Gln Ala Phe Arg Leu Gly
435 440 445

Tyr Ala Ala Leu Asp Glu Gly Gln Ile Ala Arg Ala Val Glu Ile Leu
450 455 460

Ala Arg Ser Phe Pro Gly
465 470

<210> 15
<211> 35
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<213> Artificial sequence

<220>
<223> primer

<400> 15
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<210> 16
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 16
agaggagagt tagagcctta tgaaatgcta gcagcct 37

<210> 17
<211> 35
<212> DNA
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<220>
<223> primer

<400> 17
ggtattgagg gtcgcatgtt cgacgccctc gcccg 35

<210> 18
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<212> DNA
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<220>
<223> primer

<400> 18
agaggagagt tagagcctca gagactggtg aacttgc 37

<210> 19
<211> 35
<212> DNA
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<220>
<223> primer

<400> 19
ggtattgagg gtcgcatgga acatttgctg aatcc 35

<210> 20
<211> 37
<212> DNA
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<220>
<223> primer

<400> 20

agaggagagt tagagcctta aacgccgttg tttatcg 37

<210> 21
<211> 35
<212> DNA
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<220>
<223> primer

<400> 21
ggtattgagg gtcgcatgcg cgagcctctt gccct 35

<210> 22
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 22
agaggagagt tagagcctca gccggggaag ctccggg 37

<210> 23
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 23
ggtattgagg gtcgcatgtc cacgcaggcg gccat 35

<210> 24
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 24
agaggagagt tagagcctca ctcacgattc acattgc 37

<210> 25
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 25
ggtattgagg gtcgcatgcc agaattagct aatga 35

<210> 26
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 26
agaggagagt tagagcctta ttcgtcctct tgtaaaa 37

<210> 27
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 27
ggtattgagg gtcgcatgcg ctctacgacg gctcc 35

<210> 28
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 28
agaggagagt tagagcctca gccgcgcagc accttgg 37

<210> 29
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 29
ggtattgagg gtcgcatgtt tgagaacatt accgc 35

<210> 30
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 30
agaggagagt tagagcctta cagcactgcc acaatcg 37

<210> 31
<211> 1194
<212> DNA
<213> E. coli

<400> 31
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aaagaagacc ctcgcagcga caaagtgaat ttaagtatcg gtctgtacta caacgaagac 120

ggaattattc cacaactgca agccgtggcg gaggcggaag cgcgcctgaa tgcgcagcct 180

catggcgctt cgctttattt accgatggaa gggcttaact gctatcgcca tgccattgcg 240

ccgctgctgt ttggtgcgga ccatccggta ctgaaacaac agcgcgtagc aaccattcaa 300

acccttggcg gctccggggc attgaaagtg ggcgcggatt tcctgaaacg ctacttcccg 360

gaatcaggcg tctgggtcag cgatcctacc tgggaaaacc gcgtagcaat attcgccggg 420

gctggattcg aagtgagtac ttacccctgg tatgacgaag cgactaacgg cgtgcgcttt 480

aatgacctgt tggcgacgct gaaaacatta cctgcccgca gtattgtgtt gctgcatcca 540

tgttgccaca acccaacggg tgccgatctc actaatgatc agtgggatgc ggtgattgaa 600

attctcaaag cccgcgagct tattccattc ctcgatattg cctatcaagg atttggtgcc 660

ggtatggaag aggatgccta cgctattcgc gccattgcca gcgctggatt acccgctctg 720

gtgagcaatt cgttctcgaa aattttctcc ctttacggcg agcgcgtcgg cggactttct 780

gttatgtgtg aagatgccga agccgctggc cgcgtactgg ggcaattgaa agcaacagtt 840

cgccgcaact actccagccc gccgaatttt ggtgcgcagg tggtggctgc agtgctgaat 900

gacgaggcat tgaaagccag ctggctggcg gaagtagaag agatgcgtac tcgcattctg 960

gcaatgcgtc aggaattggt gaaggtatta agcacagaga tgccagaacg caatttcgat 1020

tatctgctta atcagcgcgg catgttcagt tataccggtt taagtgccgc tcaggttgac 1080

cgactacgtg aagaatttgg tgtctatctc atcgccagcg gtcgcatgtg tgtcgccggg 1140

ttaaatacgg caaatgtaca acgtgtggca aaggcgtttg ctgcggtgat gtaa 1194

<210> 32
<211> 397
<212> PRT
<213> E. coli

<400> 32

Val Phe Gln Lys Val Asp Ala Tyr Ala Gly Asp Pro Ile Leu Thr Leu
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Met Glu Arg Phe Lys Glu Asp Pro Arg Ser Asp Lys Val Asn Leu Ser
20 25 30

Ile Gly Leu Tyr Tyr Asn Glu Asp Gly Ile Ile Pro Gln Leu Gln Ala
35 40 45

Val Ala Glu Ala Glu Ala Arg Leu Asn Ala Gln Pro His Gly Ala Ser
50 55 60

Leu Tyr Leu Pro Met Glu Gly Leu Asn Cys Tyr Arg His Ala Ile Ala
65 70 75 80

Pro Leu Leu Phe Gly Ala Asp His Pro Val Leu Lys Gln Gln Arg Val
85 90 95

Ala Thr Ile Gln Thr Leu Gly Gly Ser Gly Ala Leu Lys Val Gly Ala
100 105 110

Asp Phe Leu Lys Arg Tyr Phe Pro Glu Ser Gly Val Trp Val Ser Asp
115 120 125

Pro Thr Trp Glu Asn Arg Val Ala Ile Phe Ala Gly Ala Gly Phe Glu
130 135 140

Val Ser Thr Tyr Pro Trp Tyr Asp Glu Ala Thr Asn Gly Val Arg Phe
145 150 155 160

Asn Asp Leu Leu Ala Thr Leu Lys Thr Leu Pro Ala Arg Ser Ile Val
165 170 175

Leu Leu His Pro Cys Cys His Asn Pro Thr Gly Ala Asp Leu Thr Asn
180 185 190

Asp Gln Trp Asp Ala Val Ile Glu Ile Leu Lys Ala Arg Glu Leu Ile
195 200 205

Pro Phe Leu Asp Ile Ala Tyr Gln Gly Phe Gly Ala Gly Met Glu Glu
210 215 220

Asp Ala Tyr Ala Ile Arg Ala Ile Ala Ser Ala Gly Leu Pro Ala Leu
225 230 235 240

Val Ser Asn Ser Phe Ser Lys Ile Phe Ser Leu Tyr Gly Glu Arg Val
245 250 255

Gly Gly Leu Ser Val Met Cys Glu Asp Ala Glu Ala Ala Gly Arg Val

260 265 270

Leu Gly Gln Leu Lys Ala Thr Val Arg Arg Asn Tyr Ser Ser Pro Pro
275 280 285

Asn Phe Gly Ala Gln Val Val Ala Ala Val Leu Asn Asp Glu Ala Leu
290 295 300

Lys Ala Ser Trp Leu Ala Glu Val Glu Glu Met Arg Thr Arg Ile Leu
305 310 315 320

Ala Met Arg Gln Glu Leu Val Lys Val Leu Ser Thr Glu Met Pro Glu
325 330 335

Arg Asn Phe Asp Tyr Leu Leu Asn Gln Arg Gly Met Phe Ser Tyr Thr
340 345 350

Gly Leu Ser Ala Ala Gln Val Asp Arg Leu Arg Glu Glu Phe Gly Val
355 360 365

Tyr Leu Ile Ala Ser Gly Arg Met Cys Val Ala Gly Leu Asn Thr Ala
370 375 380

Asn Val Gln Arg Val Ala Lys Ala Phe Ala Ala Val Met
385 390 395

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<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 33
ggtattgagg gtcgcgtgtt tcaaaaagtt gacgc 35

<210> 34
<211> 37
<212> DNA
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<220>
<223> primer

<400> 34
agaggagagt tagagcctta catcaccgca gcaaacg 37

<210> 35
<211> 35

<212> DNA
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<220>
<223> primer

<400> 35
ggtattgagg gtcgcatgga gtccaaagtc gttga 35

<210> 36
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 36
agaggagagt tagagcctta cacttggaaa acagcct 37

<210> 37
<211> 35
<212> DNA
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<220>
<223> primer

<400> 37
ggtattgagg gtcgcatgaa aaactggaaa acaag 35

<210> 38
<211> 37
<212> DNA
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<220>
<223> primer

<400> 38
agaggagagt tagagcctta cagcttagcg ccttcta 37

<210> 39
<211> 35
<212> DNA

<213> Artificial sequence

<220>
<223> primer

<400> 39
ggtattgagg gtcgcatgcg aggggcatta ttcaa 35

<210> 40
<211> 36

<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 40
agaggagagt tagagcctca gcccttgagc gcgaag 36

<210> 41
<211> 1416
<212> DNA
<213> E. coli

<400> 41
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cgtaccaccc gcgcttatcg tgaagaggca attattaaat ccggtatgaa cccgttcctg 120

ctggatagcg aagatgtttt tatcgattta ctgaccgaca gcggcaccgg ggcggtgacg 180

cagagcatgc aggctgcgat gatgcgcggc gacgaagcct acagcggcag tcgtagctac 240

tatgcgttag ccgagtcagt gaaaaatatc tttggttatc aatacaccat tccgactcac 300

cagggccgtg gcgcagagca aatctatatt ccggtactga ttaaaaaacg cgagcaggaa 360

aaaggcctgg atcgcagcaa aatggtggcg ttctctaact atttctttga taccacgcag 420

ggccatagcc agatcaacgg ctgtaccgtg cgtaacgtct atatcaaaga agccttcgat 480

acgggcgtgc gttacgactt taaaggcaac tttgaccttg agggattaga acgcggtatt 540

gaagaagttg gtccgaataa cgtgccgtat atcgttgcaa ccatcaccag taactctgca 600

ggtggtcagc cggtttcact ggcaaactta aaagcgatgt acagcatcgc gaagaaatac 660

gatattccgg tggtaatgga ctccgcgcgc tttgctgaaa acgcctattt catcaagcag 720

cgtgaagcag aatacaaaga ctggaccatc gagcagatca cccgcgaaac ctacaaatat 780

gccgatatgc tggcgatgtc cgccaagaaa gatgcgatgg tgccgatggg cggcctgctg 840

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catgtgaaag agaacgcggc gaatattaaa ggattaacct ttacgtacga accgaaagta 1380

ttgcgtcact tcaccgcaaa acttaaagaa gtttaa 1416

<210> 42
<211> 1371
<212> DNA
<213> Citrobacter freundii

<400> 42
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cgtgatgaac gcctcaagaa aatgcaggaa gcgggttaca atactttcct gttaaattcg 120

aaagatattt atattgacct gctgacagac agtggcacta acgcaatgag cgacaagcag 180

tgggccggaa tgatgatggg tgatgaagcg tacgcgggca gcgaaaactt ctatcatctg 240

gaaagaaccg tgcaggaact gtttggcttt aaacatattg ttccgactca ccaggggcgt 300

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gttcgtgacg aagcgcacga tgccggtctg aatattgcgt ttaaaggtga tatcgatctt 480

aaaaaattac aaaagctgat tgatgaaaaa ggcgcagaga atattgcgta tatctgcctg 540

gcggtgacgg ttaacctcgc gggcggccaa ccggtctcga tggctaacat gcgtgcggtg 600

cgtgaactga cagaagcgca tggcattaaa gtgttctacg acgctacccg ctgcgtagaa 660

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ttcccggcac aaagtctggc tgccagcatc tatgtggaaa ccggcgtgcg cagtatggag 1140

cgcggaatta tctctgcggg ccgtaataac gtgaccggtg aacaccacag accgaaactg 1200

gaaaccgtgc gtctgactat tccacgtcgt gtttatacct acgcacatat ggatgttgtg 1260

gctgacggta ttattaaact ttaccagcac aaagaagata ttcgcgggct gaagtttatt 1320

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<210> 43
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 43
ggtattgagg gtcgcatgga aaactttaaa catct 35

<210> 44
<211> 37
<212> DNA
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<220>
<223> primer

<400> 44
agaggagagt tagagcctta aacttcttta agttttg 37

<210> 45
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 45
ggtattgagg gtcgcatgaa ttatccggca gaacc 35

<210> 46
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 46
agaggagagt tagagcctta gatgtaatca aagcgtg 37

<210> 47
<211> 31
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 47
ccagggcacc ggcgcagagc aaatctatat t 31

<210> 48
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 48
tgcgccggtg ccctggtgag tcggaatggt 30

<210> 49
<211> 32
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 49
tcctgcacgc ggcaaagggt tctgcactcg gt 32

<210> 50
<211> 30
<212> DNA
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<220>
<223> primer

<400> 50
ctttgccgcg tgcaggaagg cttcccgaca 30

<210> 51
<211> 29
<212> DNA
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<220>
<223> primer

<400> 51
aggggaccgg cgcagaaaac ctgttatcg 29

<210> 52
<211> 32
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 52
tctgcgccgg tcccctggtg agtcggaaca at 32

<210> 53
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Ala Gly Lys Gln Val Ser Gly Thr Ala Val Thr Val Leu Leu Gln Pro
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Gly Asp Asn Trp Met Met His Val Ala Ala Glu Gln Ile Gln Pro Gly
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Asp Ile Val Val Ala Ala Val Thr Ala Glu Cys Thr Asp Gly Tyr Phe
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Ile Ile Asp Ala Gly Val Arg Asp Val Lys Thr Leu Gln Glu Met Asp
115 120 125

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US2004/027454

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23L2/60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 128 164 A (VAN WYK PIETER J ET AL) 7 July 1992 (1992-07-07) cited in the application column 13, line 20 - column 14, line 27; claims	1,2,7,8, 24,37-45
X	PATENT ABSTRACTS OF JAPAN vol. 2002, no. 06, 4 June 2002 (2002-06-04) & JP 2002 060382 A (AJINOMOTO CO INC), 26 February 2002 (2002-02-26) abstract	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 2004	10/01/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lepretre, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/027454

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VLEGGAAR R ET AL: "Structure elucidation of monatin, a high-intensity sweetner isolated from the plant schlerochiton ilicifolius" JOURNAL OF THE CHEMICAL SOCIETY, PERKIN TRANSACTIONS 1, CHEMICAL SOCIETY. LETCHWORTH, GB, vol. 22, no. 1, 1992, pages 3095-3098, XP002968911 ISSN: 0300-922X	
A,P	WO 03/091396 A (CARGILL INC) 6 November 2003 (2003-11-06) the whole document	1-57

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US2004/027454

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US 5128164 A	07-07-1992	CH 675243 A5	14-09-1990
		DE 3820166 A1	29-12-1988
		GB 2205834 A ,B	21-12-1988
		JP 1025757 A	27-01-1989
		JP 2522689 B2	07-08-1996
		US 4975298 A	04-12-1990
		ZA 8804220 A	22-02-1989
JP 2002060382 A	26-02-2002	NONE	
WO 03091396 A	06-11-2003	WO 03091396 A2	06-11-2003
		US 2004063175 A1	01-04-2004